LITERATURE REVIEW
RENIBACTERIUM SALMONINARUM,
THE CAUSATIVE AGENT OF BACTERIAL KIDNEY DISEASE

PELTON ROUND BUTTE HYDROELECTRIC PROJECT
FERC No. 2030

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INTRODUCTION

Renibacterium salmoninarum is a bacterial fish pathogen with a host range including all salmonids and some salt water fishes. Bacterial kidney disease (BKD) is often a chronic or latent disease unless conditions permit frank disease occurrence. It was originally named Dee disease in the first report of it to the Furunculosis Committee in 1933. Atlantic salmon (*Salmo salar)* in the Dee and Spey rivers of Scotland were observed with kidney pustules. It was first detected in North America in 1935 in both brook (*Salvelinus fontinalis*) and brown trout (*Salmo trutta*) in Massachusetts (Belding and Merill 1935). Prior to classification of the bacterium, it was often referred to as corynebacterial kidney disease (Post, 1987). Bacterial kidney disease is widespread and is difficult to control. The chronic nature of the disease makes estimation of prevalence difficult (Sanders et al. 1992). Also the transition to salt water of infected anadromous fish may cause 45 per cent mortality to these stocks, but it often goes unrecognized as a major component in losses to salmonid fisheries (Banner et al. 1983 and 1986, Sanders et al. 1992).

GEOGRAPHIC DISTRIBUTION

First observed in Atlantic salmon in Scotland, it is now reported in salmon and trout in both the northern and southern hemisphere and was probably spread along with transplants of these species over the past century (Moffitt 1990). Bacterial kidney disease has been found throughout Europe (Post 1987). It was detected in hatchery fish in Japan in 1973 (Kimura 1978). *Renibacterium salmoninarum* antigen has been detected among wild chum salmon (*Oncoryhnchus keta*) populations in Japan. Antigen was detected in 5% of the chum salmon, although they did not have clinical signs of bacterial kidney disease (BKD) (Sakai and Kobayashi 1992). The pathogen is probably endemic in most, if not all, of the salmonids of North America. The antigen for *R. salmoninarum* is found at some level in all stocks of salmonids examined in Oregon. In the southern hemisphere it has been detected in salmonid fish stocks in Chile (Fryer and Sanders 1981, Gutenberger 1993).

SUSCEPTIBLE SPECIES

It appears that all salmonids can be infected with *R. salmoninarum*. Brook trout are the most susceptible and rainbow trout (*O. mykiss*) appear to be the most resistant. Clinical cases of BKD have not been detected in mountain whitefish (*Prosopium williamsoni*) or graylings (*Thymallus arcticus*). Low to high levels of BKD antigen have been detected mountain whitefish from the Deschutes River in Oregon and graylings from Alaska by BKD Enzyme Linked

Immunosorbent immunoassay (ELISA) methods. Variations in the susceptibility of certain salmonid stocks has been attributed to possible differences in the speed of macrophage responses (Wither and Evelyn 1990, Gutenberger 1993). Freshwater fishes other than salmonids have not been detected with infections of this pathogen; although infected fish from salt water have been reported. Sable fish have been reported with BKD by Sakai (1992).

Recent studies have surveyed a variety of other aquatic organisms for the presence of *R. salmoninarum* antigen The results are intriguing. The question remaining is; is the presence of detectable antigen evidence of infection? The detection of *R. salmoninarum* antigen in the aquatic environment of pen-cultured coho salmon (*O. kisutch*) was attempted. Flounder (*Limanda herzensteini*), greenling (*Hexagrammos otakii),* Japanese sculpin (*Cottus japonicus*), and flathead (*Platycephalus indicus*) captured by fishing around coho salmon net pens were examined for the presence of *R. salmoninarum* antigen by an indirect dot blot assay and by an indirect fluorescent-antibody technique using polyclonal and monoclonal antibodies. *R.einbacterium salmoninarum* antigen was detected from kidney samples of six flathead. Also, 86 scallops (*Patinopecten yessoensis*) were hung from the edge of the net pen for 50 days, and *R. salmoninarum* antigen was demonstrated in 31 samples by the indirect dot blot assay and the indirect fluorescent-antibody technique (Sakai and Kobayashi 1992).

Another study was not able to detect infection by BKD in these other potential hosts. The blue mussel (*Mytilus edulis*) commonly fouls netpens on salmonid farms, and salmon farmers have expressed concerns that this bivalve may serve as a source of infectious agents for penned salmonids. The chief concern was that the mussel might concentrate the bacterial kidney disease agent from the water column. The water is contaminated with the feces of infected salmon. The bivalves could then serve as a continuous source of infections with this pathogen. Results showed that the mussel is capable of rapidly clearing sea water of suspended BKD cells. In the process, most or all of the ingested bacterial cells were killed by the mussel (Paclibare et al. 1994).

ETIOLOGICAL AGENT — TAXONOMY AND VIRULENCE FACTORS

The bacterium is a small gram positive rod shaped organism, that often occurs in pairs. It is non-acid fast, non motile with dimensions of 0.4 by 0.8 micrometers. Growth occurs between 4 to 22°C with an optimum of 15°C. Only a single serotype has been identified. The bacterium causing bacterial kidney disease was of uncertain taxonomic status for some time. It was designated to the genus *Corynebacterium* but no species was assigned to it. In 1978 it was classified as *Corynebacterium salmoninus* by Sanders and Fryer. However soon thereafter, Sanders and Fryer (1980) reassigned the bacterium to a novel group, *Renibacterium salmoninarum.* based on chemical composition of the cell wall and DNA characteristics.

Taxonomy

Recent taxonomic investigations utilizing molecular techniques have further refined the definition of this organism (Banner et al. 1991, Gutenberger et al. 1993). The guanine plus cytosine, G +C, content of *R. salmoninarum* was most recently determined to be 55.5 ± 0.43% by Banner and co-workers (1991). This DNA analysis placed the bacterium just on the lower limit of the high G + C content organisms. The cell wall analysis performed by Sanders and Fryer (1980) removed this organism from consideration as being related to the *Corynebacterium* genus.

The ribosomal RNA (rRNA) sequence data is one of the most useful pieces of information in taxonomy. A nearly complete sequence of the 16S rRNA of *R. salmoninarum* was determined and used to compare it to other 17 genera of gram positive bacteria. This comparison revealed that *R. salmoninarum* was most closely related to the high G+C actinomycetes bacteria. A closer examination showed that it was clustered with the soil bacterium *Terabacter tumescens, Streptomyces coelicolor* and *Nocardiodes luteus*. Final analysis produced a very close match to *Arthrobacter globiformis* and bacterium of soil and silage. Many other biochemical and physiological characteristics are also common to the two organisms (Gutenberger 1993). Thus, *R. salmoninarum* remains a unique bacterium possibly evolving from a soil bacterium to a fastidious pathogen. The study by Banner and co-workers (1991) corroborated this evidence that the bacterium is genetically more closely linked to high G + C content bacteria.

Renibacterium salmoninarum requires a reducing media containing L-cysteine or L-cystine for growth. However, D-cysteine can not replace the L- enantiomer indicating a specific metabolic requirement for the amino acid. It was first isolated in 1935 on nutrient agar containing defibrinated blood (Belding and Merill 1935). Later a media was developed that appeared to be more complete in satisfying the growth requirements of the organism by Ordal and Earp (1956) that used cysteine and human blood. The most common medium used in the isolation of BKD presently is the improved kidney disease medium (KDM-2) that uses bovine serum. The requirement for whole blood could be circumvented using blood serum alone (Evelyn 1977). Meuller-Hinton medium with 0.1% cysteine-HCl is also used to grow the bacterium. Somewhat acidic conditions of pH 6.5 is considered by some researchers to be optimum (Post 1987). It does not utilize carbohydrates.

This bacterium is very slow growing even on these enriched media. Growth takes from five to 18 days at 15°C. The generation time is 24 hours at 15°C. Colonies appear round, very convex, smooth to glistening white with complete margins. Diagnostic laboratories continue incubation for prolonged periods of up to three months if BKD is suspected.

Virulence

The virulence of *R. salmoninarum* has been associated with a variety of factors and characteristics. One of the initial characteristics noted was that the organism was often seen intracellularly in phagocytes and was small in size. Difficulty in *in vitro* cultivation and the need for growth factors was also an indication of the intracellular nature of this pathogen (Earp 1953). The heat stable, soluble extracellular protein designated p57 has been implicated in the ability of *R. salmoninarum* to replicate within macrophages and other cells (Rockey et al. 1991). It has also shown to be responsible for the organisms hydrophobic nature (Bruno 1988). Antibody suppression may also be one of its functions (Turaga et al. 1987). The protein has hemagglutinating properties with rabbit erythrocytes (Weins and Kaattari 1991).

Other factors involved in allowing intracellular replication are the strong cell wall, a catalase, which inactivates hydrogen peroxide from macrophages, and resistance to acidic conditions. The hydrophobicity of the bacterial cell wall allows phagocytosis by macrophages. The slow replication process may also aid in intracellular growth and survival. Slower growth allows the BKD organism to resist antibiotics and the defensive enzymes of the host (Gutenberger et al. 1991).

Among the other virulence factors is the hydrophobicity of the organism associated with high iron content; although little is known of the means by which iron is acquired. Seven strains of *R. salmoninarum* were grown in iron-restricted media. A common means of obtaining iron is through specific high affinity molecules called siderophores. Siderophore production, however, was not detected by chemical assays. The growth of all strains, but particularly non-hydrophobic strains, was considerably reduced in the presence of the high-affinity iron chelators or when cultured in Chelex-treated medium. This indicated the requirement of iron for growth. The ability of the BKD strains to gain and retain iron was shown in competitive binding studies. Culture supernatant from both hydrophobic and nonhydrophobic strains was found to inhibit the binding of iron by bovine transferrin, and this inhibition was most pronounced in supernatants derived from iron-sufficient cultures. A strong iron reductase activity was detected in *R. salmoninarum* cells. This suggests that iron reductase is an important component of the iron acquisition mechanism of *R. salmoninarum* (Grayson et al. 1995).

DISEASE SIGNS

The number of fish exhibiting external signs of BKD may vary from a few to greater than 50% of the population. Only minimal gross pathology is occasionally seen even during the later stages of the disease. Early signs may be only behavioral listlessness and lethargic swimming. Some fish may appear dark. Some infected fish will swim to the sides because of the infrequent

loss of sight. Others may have no external signs. In other cases, gross signs are observed. Abscesses appear under the skin and often rupture into the water. Exopthalmia also occurs frequently. Behind the eyes, edematous fluid and granulatious tissue have formed. Malnourishment occurs because the fish are blind and cannot feed (Post 1987 and Gutenberger 1993). Swelling of the abdomen and blister like lesions are occasionally seen. Extremes in water temperature, secondary or mixed infection, high population densities and change to salt water environment have all been suggested as factors in transition of the disease to an acute phase from the latent state (Gutenberger 1993). The disease is protracted at low temperatures with a high cumulative mortality. At higher temperatures the disease may be more acute but with a lower cumulative mortality (Fryer, 1979).

Internally abscesses of white to gray color are evident on one or more of the organs. Kidneys show the abscesses frequently but BKD may be found in many other locations within the fish. The abscesses are filled with a purulent mixture of bacteria, dead tissue cells, blood and debris. Focal lesions are also found in the spleen, liver, and most other organs. The affected organs are often swollen and an opaque pseudomembrane may cover them (Fryer and Sanders 1981). Little body fat remains in the later stages of disease during protracted illness (Gutenberger 1993).

Histological reports suggest that BKD is similar to glomerulonephritis in mammals, because of the damage to glomeruli and renal tubules (Young and Chapman 1978). Heart involvement is also seen in the later stages with the invasion of infected phagocytes. Most tissues are thought to be damaged from extracellular products released by the bacterium or infected macrophages (Bruno 1986, Gutenberger 1993). However, there is little damage from the extracellular products alone. Pathology is seen only in the presence of the bacterial cells (Banner ODFW Fish Pathology, personal communication).

A study of coho salmon (*O. kisutch*) experimentally infected with *R. salmoninarum* noted histopathological changes occur in the renal and splenic haemopoietic tissues. In both tissues, different grades in the progression of the infection can be distinguished. The grades of infection can be characterized according to the location of the bacteria and the tissue injuries. Ultrastructural observation has revealed sinusoidal cells, macrophages, and reticular and barrier cells to be infected by the pathogen, and necrosis of the tissue to be general in advanced stages of the infection. Despite destruction of the haemopoietic tissue, plasmacytopoietic foci have been frequently observed in both organs. Other major changes are in the appearance of epithelioid cells and an increase in the number of barrier cells. These two cell types might be involved in the local defense response to the pathogen, but they may also act as a reservoir and proliferation locus for *R. salmoninarum* (Flano et al. 1996).

Many infections with *R. salmoninarum* are asymptomatic but chronic. This chronic state may exist because of an ineffective or non-existent immune response. A study that explored the immune state of BKD infected fish was undertaken. Challenge of Atlantic salmon by intraperitoneal injection with *R. salmoninarum* at low temperature (7-8°C) caused the fish to become asymptomatically infected. Although no overt clinical signs appeared and no deaths occurred for 14 weeks postinfection, most live fish had low levels of *R. salmoninarum*, as indicated by colony-forming units (cfu) per gram of kidney tissue cultured. Only 5-6% of kidney samples from one group and none from the second group of fish tested positive for *R. salmoninarum* soluble antigens by Western blot. Although the fish holding temperature was increased to 10-11°C on week 13 postinfection, only a low number of fish died from weeks 15 to 29. By weeks 27 and 31 postinfection, no live sampled fish had detectable *R. salmoninarum* by kidney tissue cultured. At 31 weeks postinfection, only 4% of live sampled fish from the two tanks produced antibody responses to the *R. salmoninarum* soluble antigens at levels detectable by electroimmunotransfer blot (EITB). Analysis of commercially reared Atlantic salmon from the Bay of Fundy demonstrated that 32% of the fish were asymptomatically infected, but Western blot and EITB results for these fish were similar to those for the low-temperature experimental challenge fish (Lovely et al. 1994).

TRANSMISSION

Renibacterium salmoninarum can be transmitted vertically (i.e. from parent to progeny) and horizontally (i.e. from fish to fish). Oral transmission has been demonstrated by feeding infected tissue of salmon or trout to salmonids (Wood and Wallis 1956, Ordal and Earp 1956). Transmission has also been noted through external wounds on susceptible fish in water containing bacteria (Warren 1991). Vertical transmission occurs from mother fish to young by the presence of the bacteria within the egg. Surface disinfection of eggs with povidone-iodine does not eliminate the bacteria from the egg. Many of the juveniles from these surface sterilized eggs are infected with *R. salmoninarum* (Evelyn et al. 1986). Water chemistry has also been implicated as a factor in transmission. The disease appears to be more prevalent under soft water conditions (Post 1987).

The major source of disease seems to be chronically or latently infected carrier fish. These fish continually release the bacterium in their feces. Fecal material from infected fish will have viable bacteria for up to 21 days in fresh water (Austin and Rayment 1985). Lesions on the skin of such fish will rupture and release infectious bacteria. Prior to the pasteurization of salmon for fish feed, *R. salmoninarum* was being fed directly to young in fish culture facilities (Fryer and Sanders 1981). Infected fish in the water supplies of fish culture facilities also represent a source of infection. These all represent patterns of horizontal transmission of the bacterium.

In culture facilities such as sea pen rearing farms, horizontal transmission of BKD is an important consideration. The fecal-oral route of horizontal transmission among farmed salmon held in sea water was investigated. Horizontal transmission probably explained the significant increase in prevalence of BKD observed within a regularly sampled population of chinook salmon (*O. tshawytscha*) held in a sea water netpen. Viable BKD bacteria were found in the sea water sampled from within a netpen of BKD-affected chinook. Once shed into sea water it survives long enough to be ingested by neighboring fish. The feces from these fish appeared to be the source of BKD bacterium in the sea water. Survival experiments revealed that *R. salmoninarum* remained viable in sea water for up to one week. A fecal-oral route of horizontal transmission was demonstrated by orally intubating *R. salmoninarum* -laden feces into young coho salmon (*O. kisutch*). The *R. salmoninarum* intubated group experienced significantly higher BKD-related mortality than the control group. This indicated that the fecal-oral route of horizontal transmission may contribute significantly to the increasing prevalence of BKD in farmed salmon (Balfry et al. 1996).

Horizontal transmission has also been established by various other methods in laboratory challenges. Two challenge methods for inducing BKD in salmon were evaluated using chinook salmon. The first method involved immersing the fish in various concentrations of *R. salmoninarum* cells (10^4 to 10^6/ml) for 15 or 30 minutes. The second method was based on cohabitation of uninfected fish with fish previously injected intraperitoneally with various doses (10^3, 10^5 and 10^7) of *R. salmoninarum* cells. Both methods were successful in establishing BKD in the test fish (Murray et al. 1992).

Vertical transmission has been established by various studies. One possible way in which *R. salmoninarum* is able to maintain itself in eggs was suggested to be its resistance to lysozyme. The yolk material of coho salmon eggs contains the naturally occurring antibacterial factor, lysozyme. The antibacterial properties of this lysozyme were studied with selected bacterial fish pathogens. It was found to be rapidly bactericidal to *Aeromonas hydrophila, A. salmonicida,* and *Carnobacterium piscicola* at a concentration of 700 µg/ml, a concentration approximately one-third of that found in the yolk of most salmonid eggs. However, the lysis that it caused did not occur with *R. salmoninarum*. *Renibacterium salmoninarum* was not killed when incubated with as much as 1900 µg/ml of the enzyme for 90 minutes. The antibacterial role of lysozyme is to role in prevent the mother to progeny (vertical) transmission of some bacterial fish pathogens. Lysozyme's failure to kill *R. salmoninarum* may explain why this organism is readily vertically transmitted (Yousif et al. 1994).

Other studies on brood stock segregation based on ELISA testing of adult chinook salmon have established that vertical transmission of BKD can be limited by removal of eggs from highly

positive adults (Pascho et al. 1991). Progeny from adults that tested negative or low for BKD antigen had much lower levels of BKD.

EPIZOOTIOLOGY

Loss of infected fish begins to occur in early life stages and continues throughout life. Often epizootic losses begin in culture conditions as anadromous fish begin the smoltification process. These fish enter salt water with the disease and losses continue to occur and may even be greater (Fryer and Sanders 1981, Banner et al. 1983, 1986). Individual losses in wild fish occur, but epizootic outbreaks have not been documented. The transmission rate may be lower in natural conditions where horizontal transmission is less likely.

The chronic nature of bacterial kidney disease is characterized by slow development. Abscesses often develop because the bacterium is non-motile and spreads more slowly through the host. Disease more often occurs during the cooler months and declines in the summer. The summer survivors of infection will often later succumb with cooler weather.

Bacterial kidney disease (BKD) is an important contributor to mortality of salmonids in hatcheries in the Columbia River basin. A study investigated the impact of BKD on the survival of downstream migrants to determine the disease-related mortality among these. The impact on juvenile salmonids was examined by determining the percentage of downriver migrants infected with *R. salmoninarum* and evaluating the effects of salt water on the progress of the disease. During the two years of study, approximately 20% of the three species of migrating hatchery and wild salmonids (*Oncorhynchus* spp.) were infected with *R. salmoninarum.* Mortality caused by BKD increased when fish were held in salt water (Sanders et al. 1992).

Subgroups of fish from broodstock segregation studies (Pascho et al. 1991) were marked with passive integrated transponder (PIT) tags and monitored by PIT-tag detectors during the first 342 km of their migration to the Pacific Ocean. Differences in the recovery of tagged fish were significant. The data suggested that in-river survival was higher in the progeny group from parents that had low *R. salmoninarum* infection levels or tested negative for *R. salmoninarum* than in the group from female parents with high infection levels (Pascho et al. 1993).

DETECTION METHODS

Initial detection has been from obvious disease signs and observation of the bacterium taken from lesions. The presence of gram positive small rods or diplobacilli from lesions is a presumptive diagnosis for BKD. Histopathology of lesions reveal distinctive characteristics which add in the diagnosis. Isolation of the bacterium on any of the selectively enriched media for *R.*

salmoninarum growth is a definitive diagnosis of BKD. Since no other similar pathogenic bacteria are found in salmonid fish, this level of diagnosis is sufficient in most cases of clinical disease.

Latently infected or carrier fish require additional testing to confirm the presence of *R. salmoninarum*. Stained preparations of suspected organs or direct fluorescent antibody tests (DFAT) are necessary. The DFAT is sensitive and quick in comparison to the direct observation of gram stained organ squashes (Civitanich 1994). The drop plate technique has been used with tissues for direct cultures. This technique requires up to three weeks to allow growth of the bacterium from the homogenized tissues. A classical immunological technique, immunodiffusion is rarely used now. The reaction with the antibody is relatively specific, though cross reacting antigens in other organisms lead to false positive results (Post 1987). Recently the enzyme linked immunoabsorbent antibody assay (ELISA) has become widely used as the standard method in detecting the presence of *R. salmoninarum* antigen (the p57 protein).

Staining trials were conducted over a five-year period to explore ways of improving the direct fluorescent antibody technique for *R. salmoninarum.* In most salmonid kidney tissue smears that were pretreated with the organic solvent xylene, acetone, or methanol for two minutes, *R. salmoninarum* had substantially higher fluorescence intensity and better uniformity of fluorescence than *R. salmoninarum* in non-pretreated controls. Increasing fluorescent antibody staining time greatly increased fluorescence intensity in most trials, but did not affect uniformity of fluorescence. Improved staining facilitated *R. salmoninarum* detection and identification, allowing more reliable quantification in standardized smear preparations (Cvitanich 1994).

The development of monoclonal antibody probes (MAbs) to bacterial and parasitic fish pathogens has provided useful tools for the rapid immunodiagnosis and control of disease. Immunohistochemistry, IFAT and ELISA tests have been developed to detect a variety of infectious agents, including those causing bacterial kidney disease, mycobacteriosis (*Mycobacterium* spp.) and proliferative kidney disease (PKX) (Adams et al. 1995). A double-antibody enzyme-linked immunosorbent assay (ELISA) has been developed for the detection of *R. salmoninarum*. The ELISA assay was rapid and sensitive, with a lower limit of detection of 0.3 µg *R. salmoninarum* antigen per milliliter. Under field conditions with naturally infected fish, the ELISA was more sensitive in this study than bacteriological methods, and therefore has the potential for the rapid screening of fish from wild or cultured populations for evidence of BKD infection. However, in some cases, fish previously infected with BKD were not detected by this method (Olea et al. 1993). Most diagnosticians and researchers consider culturing the organism to bethe most sensitive and accurate means of identifying BKD infections.

The enzyme-linked immunosorbent assay (ELISA) and the fluorescent antibody test (FAT) were compared for their sensitivity in detection of *R. salmoninarum* in kidney tissues of

Alaskan salmonids. The ELISA appeared to be more sensitive in detecting infections. The FAT did not detect *R. salmoninarum* in 80% of the ELISA-positive samples but was positive for *R. salmoninarum* in 28% of the samples that were ELISA-negative. This contradiction may have been because of low-level washover of *R. salmoninarum* cells from smears containing large numbers of cells when slides containing multiple samples were rinsed in a common vessel during the FAT procedures. The FAT routinely did not detect infections in *R. salmoninarum* positive fish the tissues of which produced a mean ELISA optical density ($O.D._{540}$) value less than or equal to 0.173, and inconsistently detected infections in fish with ELISA values > 0.173 but < 0.978. The 0.978 optical density was the mean ELISA value at which the FAT routinely detected *R. salmoninarum* positive fish. Based on the ELISA results, *R. salmoninarum* occurred in only 9% of the Alaskan Pacific salmon tested in both wild and hatchery stocks. The very high stock prevalences and levels of *R. salmoninarum* antigen detected in wild trout (*Oncorhynchus* spp.), char, (*Salvelinus* spp.), and grayling (*Thymallus arcticus*) having no clinical signs of bacterial kidney disease suggest these species may be somewhat resistant hosts and important freshwater reservoirs of *R. salmoninarum* (Meyers et al. 1993).

Other immunological methods have been used for experimental diagnoses. Some of these methods may be applied to field situations. Other methods have shown the shortcomings of currently used procedures. Cell surface extracts from isolates of *R. salmoninarum*, *Corynebacterium aquaticum*, and *Carnobacterium piscicola* were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. Although the electrophoretic analysis showed that the three bacterial species displayed different protein profiles, the immunoblot procedure with rabbit sera directed against *R. salmoninarum* ATCC 33209 and C. *aquaticum* ATCC 14665 revealed an immunoreactive band of approximately 57 kilodaltons (kDa) common to all isolates. These results suggest that any immunodiagnostic procedure relying on the detection of the 57-kDa antigen of *R. salmoninarum* can result in false positive reactions (Bandin et al. 1993).

Another study of enhanced Western blot techniques was to examine the practical and specific uses of this method. Six strains of *R. salmoninarum* were examined by Western blotting using polyclonal and monoclonal antisera produced against the 57 kDa surface protein (p57 antigen). An enhanced chemiluminescence (ECL) detection system increased the sensitivity of the reaction when compared to the use of chromogenic substrates. Western blotting was not as sensitive as culturing methods for the detection of *R. salmoninarum* during early stages of infection in salmonids. Yet, Western blotting was determined to be specific to *R. salmoninarum*, as cross-reactions did not occur with *C. aquaticum* or *C. piscicola* (McIntosh and Austin 1996).

Methods for the detection of *R. salmoninarum* by polymerase chain reaction (PCR) have been described. A rapid procedure has been developed for the extraction of DNA which could be applied to infected kidney homogenates and head kidney lymphocyte preparations. One target for

DNA amplification is a 376-bp region of the gene encoding the 57-kDa major surface antigen. The PCR was specific for *R. salmoninarum* and allowed the detection of 10 to 100 cells of the pathogen. A specific size fragment of DNA is detected by gel electrophoresis. Use of this method for the examination of experimentally infected rainbow trout showed it to be as reliable as plate culture methods for the detection of *R. salmoninarum* in infected kidneys (McIntosh et al. 1996).

Other PCR methods have used dye labeled probes to detect the production of specific DNA fragments by dot blot hybridization. A specific DNA probe for the identification of *R. salmoninarum* was developed from one of three clones. The biotinylated insert probe was tested on three dilutions of DNA extracted from three strains of *R. salmoninarum* and from one strain each of *A. protophormiae, A. salmonicida, C. aquaticum, C. piscicola, Listonella anguillarum, Micrococcus luteus, Pseudomonas fluorescens, Vibrio ordalii,* and *Yersinia ruckeri.* In a dot blot assay, this probe hybridized only with the DNA from the *R. salmoninarum* strains. When used on kidney samples from fish challenged with *R. salmoninarum*, the dot blot hybridization assay with the probe was found to be as sensitive as culture methodologies. In a fluorescent antibody test, samples that were negative in culture and dot blot hybridization showed no more than one fluorescing cell in fifty microscopic fields examined. This one cell was presumed to be artifactual and represented non-specific binding of the antibodies. This DNA probe has the potential for use in the diagnosis of BKD of fish (Hariharan et al.1995).

One of the problems that has arisen with immunological assays has been the cross reactions (false positives) with other bacteria often present in samples. Polymerase chain reaction assays have been used to examine these cross reactions and to demonstrate the specificity of this assay methodology. In a study of this bacterium, tentatively identified as *Pseudomonas maltophilia*, it cross-reacted with two polyclonal antisera, one of which is used in an enzyme-linked immunosorbent assay and the other in a fluorescent antibody test to identify *R. salmoninarum*. The isolates of *P. maltophila*, and *C. piscicola* and *C. aquaticum* strains, were negative by a PCR that was designed to amplify a

segment of the gene encoding p57, a major protein of *R. salmoninarum*. These results suggest that although antibodies directed against *R. salmoninarum* cross-react with antigens of bacterial species other than *R. salmoninarum*, the cross-reacting antigen(s) is clearly not the same proteinbecause the non-*R. salmoninarum* bacteria lacked the gene encoding p57. These findings note some of the shortcomings of immunodiagnostic tests for detecting *R. salmoninarum* and indicate the high degree of specificity associated with a PCR based diagnostic technique (Brown et al. 1995)

THERAPY AND CONTROL

Therapy

Treatment has centered around the use of antibiotics for limited reduction in mortality of cultured fish. The first antibiotics used extensively were the sulfonamides fed over a period of 20 to 49 days, with weekly supplements during the summer. The disease was halted while under treatment, but mortality was just delayed (Sniezsko 1953). Later other antibiotics were used. Chloramphenicol, oxytetracycline, and aureomycin were tried with the same results; simply delaying mortality (Snieszko and Griffin 1955). Further study tested 16 additional antibiotics and revealed that erythromycin was most effective, although it to did not completely control the disease (Bullock et al. 1975). A recent evaluation showed that erythromycin and penicillin G are among the most effective and practical antibiotics available to treat BKD (Austin et al. 1985). Injection of prespawning female adults with antibiotics (penicillin G, erythromycin, or oxytetracycline) has been shown to be effective in reducing the transmission of BKD to progeny (DeCew 1972, Evelyn et al. 1986). Oxytetracyclin is not considered effective in Oregon state fish hatcheries (R. Holt,C. Banner,A. Amandi; Fish Pathologists ODFW, personal communication). Healthy coho salmon that had returned to spawn in a British Columbia hatchery were transferred to the laboratory where the females were infected with *R. salmoninarum* by injection. Five days later, half of the infected females were injected (via the dorsal sinus) with erythromycin and the other half (untreated controls) were injected with saline. Following spawning 28 days later, eggs were collected from five of the treated and five of the control females. The eggs were then fertilized with milt from males that appeared BKD free based on gross observation. Yolk samples from eyed eggs and alevins derived from the treated females contained erythromycin (1.0 to 1.6 µg/ml and 0.6 to 0.7 µg/ml, respectively) The same samples from the untreated females had no the antibiotic. None of the eggs or alevins from the five fish treated with erythromycin had culturable or membrane filtration-indirect fluorescent antibody assay positive *R.. salmoninarum*. In contrast, all eggs and alevins from untreated females were infected with BKD (Lee and Evelyn 1994). Utilization of erythromycin at Oregon State Fish hatcheries has not been shown to be this effective, but does reduce the level of infection (ODFW Fish Pathologists, personal communication).

Research has shown that erythromycin, an antibiotic commonly used for human disease, is particularly effective against *R. salmoninarum* in salmon and currently is in Investigational New Animal Drug (INAD) trials (Moffit 1990). Bandin and co-workers (1993) found erythromycin and oxytetracycline the most effacious drugs in an *in vitro* study of *R. salmoninarum* control. Sarafloxacin, a fluoroquinolone, acts intracellularly but seems to be only somewhat effective against *R. salmoninarum.* The efficacy of enrofloxacin (Baytril registered , Bayer) was evaluated

for control of *R. salmoninarum* infection in salmonids. Minimum inhibitory concentration studies indicated efficacy at 0.25-0.5 µg/mL. In laboratory challenge studies with rainbow trout (*O. mykiss*), mortality of fish receiving enrofloxacin daily at a dosage of 1.25-2.5 mg/kg for 10 days was significantly lower than that of nonmedicated fish. A general trend of increased percent survival with increasing dose was also observed (Hsu et al. 1994). The use of antibiotics will continue until such time an effective alternative therapy is developed.

Control

Control methods now being employed utilize culling and segregation of eggs from highly positive BKD females. Previously suggested methods have focused on dietary composition such as vitamins and minerals. Of these, only the addition of fluorine and iodine have had an effect on reducing disease and mortality (Elliott et al. 1989). The effects of these constituents are minor.

A useful strategy for reducing the level of BKD in hatchery or cultured fish is culling or segregation of eggs from maternal parents with high levels of *R. salmoninarum* antigen. A study of the effect of maternal *R. salmoninarum* infection levels on the prevalence and levels of BKD in progeny fish was conducted. Spring chinook salmon, were screened for *R. salmoninarum* by ELISA. On the basis of ELISA testing of kidney tissues from all fish and the testing of ovarian fluid samples from a subsample of the females by a direct membrane filtration fluorescent antibody technique (MF-FAT), selected egg lots were segregated into two groups. One group contained egg lots from male and female parents that had low *R. salmoninarum* infection levels or tested negative for *R. salmoninarum* (low-BKD group), and the other group contained egg lots from female parents with relatively high *R. salmoninarum* infection levels and male parents with various infection levels (high-BKD group). The progeny groups were maintained in separate rearing units supplied with untreated river water and were monitored for *R. salmoninarum* by the ELISA. The low BKD group had significantly lower levels of BKD. The results suggest that segregation of brood stock by the ELISA and the MF-FAT can be used to reduce the prevalence and levels of BKD in hatchery-reared spring chinook salmon, even in locations with open water supplies (Pascho et al. 1991).

Vaccines have been notably unsuccessful (Elliott et al. 1989). The immune response elicited by the vaccines (forms of the BKD bacterial antigens) is not protective (Hsu et al. 1994). There have been a variety of vaccines developed including; killed whole BKD cells, cellular proteins, with and without adjuvants, which have been administered by injection, immersion, feeding and hyperosmotic infiltration. The number and size of lesions is reduced in vaccinated fish. However, the pathological changes in the kidney may in fact be worse following vaccination (Kaattari et al. 1988). Though work continues in this area, a breakthrough in methodology is necessary for development of an efficacious vaccine.

HISTORY OF BACTERIAL KIDNEY DISEASE IN THE DESCHUTES WATERSHED FISH HATCHERIES AND OTHER LOCATIONS

Oak Springs Fish Hatchery

Bacterial kidney disease was found in rainbow trout brood stock many years ago. It is no longer a problem at this facility.

Round Butte Fish Hatchery

Renibacterium salmoninarum is found in both juvenile and adult spring chinook salmon. Summer steelhead trout have been found to carry BKD antigen at a level of about 11% from 1997 thrugh 1999.

Fall River Fish Hatchery

No detections at this location.

Wizard Falls Fish Hatchery

Atlantic salmon and brook trout have had detectable BKD infections. Loss of brook trout brood stock has occurred from *R. salmoninarum* infections at this location.

Lake Billy Chinook

A bull trout trapped in the forebay of Round Butte Dam with lesions on side had a clinical case of BKD. Kokanee have been found on occasion dead in the reservoir with BKD infections.

SUMMARY

The concerns with BKD remain important. The chronic persistent nature of the disease and the transmission by latently infected carriers to other wild or cultured fish make BKD one of the most significant fish health problems. The loss of anadromous fish from BKD during their migration to salt water exacerbates efforts to restore runs in the Columbia River. Treatment methods do not eliminate the organism from infected fish. Transmission occurs both horizontally in the water and vertically from within the egg. Thus surface disinfection with iodine, which has been so successful at reducing some pathogens, is ineffective. Vaccines have been particularly poor in providing protection.

Future work needs to address development of new approaches for chemotherapeutants that will act intracellularly. The elimination of *R. salmoninarum*, not just the creation of an inactive state, is necessary for true control. However, no matter how effective the antibiotic(s), development of resistance is always a concern. The constant use of antibiotics is expensive and is not an ecologically acceptable practice over the long term. Thus another front of attack must be made. The enhancement of an effective immune response that will prevent vertical transmission and lead to eradication of the latent state is necessary. Such enhancement through adjuvants and/or vaccines is in need of exploration.

These methods of approach to BKD are in need of long term research. The solutions will be difficult to acquire but will be most useful in dealing with this intractable fish pathogen.

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