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BACTERIAL KIDNEY DISEASE (BKD) IN MICHIGAN
SALMONIDS

presented by

Alaa-Eldin Abdel Mouty Mohamed Eissa

has been accepted towards fulfillment
of the requirements for the

__Ph.D.__   degree in   _____Pathology_____

Mohamed Faisal
Major Professor's Signature

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BACTERIAL KIDNEY DISEASE (BKD) IN MICHIGAN SALMONIDS

By

Alaa-Eldin Abdel Mouty Mohamed Eissa

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
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ABSTRACT

BACTERIAL KIDNEY DISEASE (BKD) IN MICHIGAN SALMONIDS

By

Alaa-Eldin Abdel Mouty Mohamed Eissa

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 salmonines worldwide. Historical records show that BKD affected Michigan's brook trout as early as 1955 (Allison 1958) and continued its spread to most of other salmonid species such as coho salmon (*Oncorhynchus kisutch*) spawners in the late sixties (MacLean and Yoder 1970) followed by a potent epizootic among the chinook salmon (*Oncorhynchus tshawytscha*) populations in Lake Michigan in the late 1980's (Holey et al. 1998). Despite the magnitude of the setback to fisheries conservation efforts in Michigan, relatively little research has been done on *R. salmoninarum*, primarily due to its slow-growing nature, which takes up to 12 weeks to obtain growth upon primary isolation. The tissue processing protocol and optimized culture technique developed in this study, has facilitated the isolation of large number of *R. salmoninarum* and achieved a remarkably shorter incubation time for primary isolation.

A sort of disagreement in results among the three used diagnostic assays (nPCR, Culture, Q-ELISA) was recorded during BKD testing of the

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feral and captive salmonid stocks for BKD, which may reflect the different phases of *R. salmoninarum* infection at the time of sampling. Further, the testing results demonstrated the presence of six patterns, with each of the patterns representing a probable stage along the course of *R. salmoninarum* infection.

Data also demonstrated that male spawner salmon play the same role played by female in transmission of *R. salmoninarum* by shedding of the bacteria and its soluble antigens in milt. Moreover, results revealed that Hinchenbrook coho strain is more susceptible to *R. salmoninarum* than the Michigan adapted coho salmon strain. Data also, supported the previous reports, which indicated that brook trout are highly susceptible to BKD. Also, data showed that prevalence of *R. salmoninarum* in the hatchery-raised brook trout is the same as wild strains residing in the water source and that Iron River brook trout are more vulnerable to *R. salmoninarum* infection than Assinica strain. Last, external parasites could played a possible role in initiation, speeding up the *R. salmoninarum* infection and BKD occurrence and mortalities is not related to changes in seasons. Finally, findings indicated that the adult parasitic sea lamprey is a new host range for *R. salmoninarum* where the bacteria were isolated from the kidneys of a number of sea lampreys from Lake Ontario in 2003 and 2004.

DEDICATION

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INTRODUCTION

Bacterial Kidney Disease (BKD), caused by the Gram-positive bacterium *Renibacterium salmoninarum* (*R. salmoninarum*), is a systemic disease that afflicts salmonid fish populations worldwide. *R. salmoninarum* is an obligate intracellular pathogen that is transmitted both horizontally (Mitchum and Sherman 1981; Bell et al 1984) and vertically (Evelyn et al. 1984, 1986). *R. salmoninarum* pathogenicity relies upon a number of extracellular proteins (ECP) that possess immunosuppressive (Turaga et al. 1987; Fredriksen et al. 1997). The ECP contain a water-soluble, cell surface, 57-kDa protein (*p57*) that has been demonstrated to be a major virulence factor of *R. salmoninarum* (Getchell et al. 1985; reviewed in Wiens and Kaattari 1999). BKD was originally known as the Dee Disease, due to its initial discovery in Atlantic salmon (*Salmo salar*) from Aberdeenshire Dee and the River Spey in Scotland in 1930 (Anonym 1933). Few years later the disease was first reported in brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) reared in a hatchery in Massachusetts, USA (Belding and Merrill 1935). Since the appearance of this initial report, the disease was reported in most of the cultured and wild salmonines from North America.

Since, the first report of BKD in brook trout yearlings from Michigan in 1955, the disease quickly spread to affect other salmonid species such as coho and chinook salmon. BKD resulted in epizootics among coho salmon spawners in 1967 (McLean and Yoder 1970), chinook salmon spawners

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during the 1980s (Holey et al. 1998) and recently harvested thousands of hatchery raised brook trout between the period from 2002 to 2003 (Eissa 2005, this dissertation). These continuous eruptions of BKD outbreaks and high prevalence of the *R. salmoninarum* among almost all the tested salmonid populations throughout this study revealed a new fact that *R. salmoninarum* is enzootic in Michigan salmonines and Great Lakes water basin.

Despite the aforementioned facts, the publications describing the status of BKD in Michigan salmonines fish species are fairly scarce (Hnath and Faisal 2005). This intensifies the needs to fill the gap between the rapid progress of BKD and research performed on BKD in Michigan. This submitted study is a trial to fill some of these gaps, by studying both the causative agent and the affected salmonid fish populations in Michigan.

In brief, chapter, 2 in this study dealt with the development of tissue processing protocol and culture technique that facilitated the isolation of large number of *R. salmoninarum* isolates within relatively short incubation time. The isolation of such large number of isolates from different salmonids, ages, strains, and locations lead to a conclusion that BKD is enzootic in Michigan.

Chapter 3 describes the analysis of different diagnostic testing patterns produced from different agreements and disagreements between results obtained by three BKD diagnostic assays (Nested PCR, Quantitative ELISA, and Culture) which allowed me to track the progress and different stages of naturally occurring *R. salmoninarum* infection in some Michigan feral salmonines. In chapter 4 the ability of both chinook and coho females and

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males to shed *R. salmoninarum* through the ovarian fluid and milt has been discussed. These data shed the light on the possible role of male spawner fish to spread the disease through shedding the pathogen via the milt. Thus, both males and females should equally utilized for BKD testing and culling procedures in weirs.

In chapters 5, the retrieved data supported the previous reports, which emphasized that brook trout are highly susceptible to *R. salmoninarum* infection. Also, this study shed the light on the possible contribution of a number of factors to development of BKD epizootics in Michigan hatcheries. Further, the study suggested a number of efficacious control strategies for stopping the BKD associated mortalities and minimizing the spread of BKD. Finally, data in chapter six demonstrated the successful isolation of *R. salmoninarum* from the kidneys of adult sea lamprey (*Petromyzon marinus*) but not from blood or any other organ, which indicated that sea lamprey, is a possible non salmonid host range for *R. salmoninarum* infection.

CHAPTER ONE

LITERATURE REVIEWS

I. Historical perspectives

Bacterial Kidney Disease (BKD), caused by the Gram-positive bacterium *Renibacterium 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 and Smith 1964) Other synonyms of the disease include Kidney Disease, Corynebacterial Kidney Disease and Salmonid Kidney Disease (Fryer and Sanders 1981). A 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

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(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, Scotland, 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).

II. The pathogen

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 *Renibacterium salmoninarum* (Sanders and

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Fryer 1980). Sequencing of the 16S ribosomal ribonucleic acid (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* (Holt et al. 2001).

2) Cell morphology

Renibacterium 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). *Renibacterium 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).

3) Isolation, culture and cultural characteristics

Renibacterium 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

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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 gm %, beef extract 0.3 gm %, NaCl 0.5 gm %, yeast extract 0.05 gm %, cysteine-hydrochloride 0.1 gm %, human blood 20 v/v and agar 1.5 gm %. 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 25µl 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 25µl 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 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, Chapter 2) suggested that

the modification of Selective KDM (SKDM) by incorporating 1 % Spent

medium into the agar enhanced the growth of the *R. salmoninarum* colonies,

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shortened the period of incubation, and minimized the growth of contaminating bacteria.

Renibacterium salmoninarum colonies are creamy (non-pigmented), shiny, smooth, round, raised, entire, and 1-2mm 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 broth 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).

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

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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.

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 homogeneous in their biochemical characteristics (Austin et al.1983; Goodfellow et al. 1985; Bruno and Munro 1986 a) 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 1986 a), 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 1986 a). 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).

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6) Antibiotic susceptibility

Renibacterium 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). *Renibacterium 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 ß and cycloheximide (Wolf and Dunbar 1959; Goodfellow et al. 1985).

7) Antigenic characteristics and virulence factors

Renibacterium 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 1986 a). 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.*

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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 leuco-agglutinating (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; O' Farrel et al. 2000). 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.

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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 (tDNA -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). Specially, Michigan isolates showed a higher variation in succinate dehydrogenase and esterase loci.

III. The disease

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

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(Evenden et al.1993), and *R. salmoninarum* strain virulence (Starliper et al. 1997).

a) 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).

b) Internal lesions

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Kidneys of affected fishes are usually swollen and exhibit white foci that

contain leucocytes, bacteria, and host cell debris (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).

c) 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

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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 (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 (Wood 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 include hypercellularity of the glomeruli, occlusion of Bowman's space by filamentous or granular deposits (Sami et al. 1992) and presence of eosinophilic granules in proximal tubules (Young and Chapman 1978). Massive myocarditis (Wood

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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).

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* (Beacham and Evelyn 1992). 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 experimentally infected. Mitchum and Sherman (1981) reported that brook trout were more susceptible to natural BKD infection than rainbow trout and brown trout.

3) Pathogenesis and immunity

a) Process of infection and pathogenesis

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Renibacterium 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; Flaño 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). *Renibacterium 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, b; Bruno and Munro 1986b).

Renibacterium 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).

Renibacterium 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

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infection. These antibody-p57 complexes may remain in tissue and contribute to tissue destructive hypersensitivity resulting in granulomas (Bruno 1986; Sami et al. 1992).

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).

b) 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-

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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 possess 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).

c) Environmental factors

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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

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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.

Effect of estuarine and salt-water environments

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

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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.

IV. Epizootiology

1) Geographical distribution

Bacterial Kidney Disease 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 and 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

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was presumptively 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 supporting 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).

2) Host range

Bacterial Kidney Disease has been reported in salmonids of the genera *Oncorhynchus*, *Salmo* and *Salvelinus* (Fryer and Sanders 1981). *Renibacterium 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 (*Heragrammos otaki*), flathead (*Platycephalus indicus*) and Pacific herring (*Glupea pallasi pallasi*) (Traxler and Bell 1988). *Renibacterium salmoninarum* antigen has also been detected in Japanese sculpin (*Cottus*

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Japonicus) and Japanese scallops (*Patinopecten yessoensis*) (Sakai and Kobayashi 1992). Recently, the organism has been isolated for the first time from the adult parasitic stage of Lake Ontario Sea Lamprey (*Petromyzon marinus*) (Eissa et al.2004).

3) Disease transmission

a) Source of infection

Renibacterium 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 orofecal route of horizontal transmission may contribute significantly to the increasing prevalence of BKD in salmonids.

b. Horizontal transmission

Renibacterium 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

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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 / gram 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

C (Murray et al. 1992).

c. 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

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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. 1986 a, b) where the pathogen is located in the yolk and is protected from surface disinfectants (Evelyn et al. 1986a,b; Bruno and Munro 1986 c). 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).

d. Fish as possible vectors and carriers

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

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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).

e. 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 salmonid fish

species. 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.*

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salmoninarum between sea lice infected and non-infected fish exists
(Richards et al. 1985; Frerichs and Roberts 1989).

f. 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 (Woods 1974).

V. Diagnosis of BKD

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),
selective KDM (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.

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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).

2) Antigen-antibody reactions

a. 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.

b. 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.*

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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 tests 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).

c. Enzyme linked immunosorbent assay (ELISA)

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Hsu et al. (1991) developed an improved monoclonal antibody based ELISA assay 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, is 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 (1.000 or more).

d. 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

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gave satisfactory results regardless of the fixation method used.

Immunohistochemistry has the advantage of visualizing *R. salmoninarum* and

the tissue alteration they cause simultaneously (Jansson et al. 1991; Evensen

et al. 1994). Immunohistochemistry 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).

3) Polymerase chain reaction (PCR)

Polymerase chain reaction (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 hundredfold

compared to a conventional PCR method (Pascho et al. 1998). The authors

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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 nested-PCR 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.

VI. 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).

Renibacterium 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 (Holt et al. 2001). 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* (Holt et al.2001). 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 (Holt et al.2001). Interestingly, all these characteristics are distinctly different from that of *Renibacterium*.

VII. Control

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 per 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

Intraperitoneal (*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 1hour 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 11mg/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.

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.

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

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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).

4) Prophylaxis

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.

2. Vaccination

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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 intraperitoneal 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

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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.

Gap of BKD knowledge in Michigan before 2002

Although BKD was virtually reported wherever salmonid fishes are present (Fryer and Sanders 1981; Bullock and Herman 1988, Klontz 1983), publications describing the status of BKD in Michigan salmonids fish species are rather scarce (Hnath and Faisal 2005). Historical records showed that BKD affected Michigan brook trout as early as 1955 (Allison 1958). Like in other parts of the USA and Europe, BKD continued its spread to the Coho salmon (*Oncorhynchus kisutch*) spawners in the late sixties (MacLean and Yoder 1970) and caused epizootics of chinook salmon (*Oncorhynchus tshawytscha*) in Lake Michigan in the late 1980's (Holey et al. 1998; Johnson and Hnath 1991). Despite these reports of BKD in Michigan, only two studies have dealt with *R. salmoninarum* isolation from infected Michigan fish. Starliper (1996) and Starliper et al. (1997) isolated and identified *R. salmoninarum* isolates from chinook salmon collected from Lake Michigan weirs in Michigan and Wisconsin. Additional investigations showed that *R. salmoninarum* affects more salmonid fish species than originally thought. For example, Jonas et al. 2002 detected *R. salmoninarum* antigens in Lake whitefish (*Coregonus clupeformis*) and bloaters (*C. hoyi*) collected from Lake

Michigan in 1997-1999. Hnath and Faisal (2005); Wright and Faisal (2005)

emphasized the fact that *R. salmoninarum* is endemic to the Great Lakes

basin and effective control measures have to be seriously pursued.   In order

to develop sound management strategies to control BKD in Michigan, several

gaps of knowledge have to be filled. This submitted study is a trial to fill some

of these gaps.

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	-	

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

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

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Criteria	Description
Motility	Non motile
Growth at 15 °C	+
Growth at 5, 22 °C	+
Growth at 0, 30, 37 °C	No growth
Mol % G+C	53
Mycolic acid	Absent
Cell wall peptidoglycan amino acids	Glutamic, lysine , alanine and glycine
Cell wall sugars	Glucose, arabinose, mannose, and
Requirement for cysteine	Required
Survival	Obligate intarcellular
Major antigenic factor	57 *kDa* protein (*P57*)
Antigenicicity	Strains appears antigenically homologous
Leucoagglutinins	Exist
Leucocytolysins	Exist
Metalloproteinase	Exist
Immune-complexes	Occurs in kidney, liver, spleen
Biochemical and genetic diversity	Biochemically uniform with very minimal genetic differences
Phylogeny	Phylogentically related to actinomycetes and closely related to *Arthrobacter* spp and *Brevibacterium*
Survival in river water	Up to 1 week
Survival in feaces and mud	Up to 21 days
Transmission	Horizontal (wounds, orofecal, gills, contact) and vertical (intraovum or egg shell contamination)

Table 2. Summary of different metabolic, antigenic and pathogenic characters of *Renibacterium salmoninarum*

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CHAPTER TWO

Primary Isolation of *Renibacterium salmoninarum* from naturally infected salmonine stocks in Michigan using a modified tissue processing protocol

ABSTRACT

Throughout the 1980s, massive dieoffs involving chinook salmon occurred in Lake Michigan. These well-publicized epizootics were attributed primarily, but not entirely, to Bacterial Kidney Disease (BKD) caused by *Renibacterium salmoninarum* (*R. salmoninarum*). Despite the magnitude of the setback to fisheries conservation efforts in Michigan, relatively little research was done on *R. salmoninarum*, primarily due to the slow-growing nature of this bacterium which takes up to four weeks to obtain a primary isolation. In this study, I developed a tissue processing protocol that collected the whole kidney of an infected fish and exposed it to high sheer stomaching. This significantly shortened the incubation time and allowed *R. salmoninarum* to be cultured with relative ease.

A total of 566 *R. salmoninarum* isolates have been retrieved from the kidneys of wild, feral, and captive fish assayed between 2002-2004 from spawning weirs and state fish hatcheries at various locations in Michigan.

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These isolates constitute a unique resource for future studies of *R. salmoninarum* and BKD in the Great Lakes.

Findings also demonstrated that *R. salmoninarum* is ubiquitous in Michigan because the pathogen was isolated from each population and lot of fish examined. Prevalence of *R. salmoninarum* and the clinical lesions associated with infection varied among species, strains of the same species, and geographic locations.

Renibacterium salmoninarum isolates were biochemically homogenous and similar to those of other *R. salmoninarum* strains isolated worldwide. Antibiogram revealed high sensitivity of *R. salmoninarum* isolates to enrofloxacin and ciprofloxacin. Interestingly, two isolates were resistant to erythromycin, which is the antibiotic of choice for treatment of BKD.

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INTRODUCTION

Due to its slow-growing nature, *Renibacterium salmoninarum* Sanders and

Fryer 1980, the causative agent of bacterial kidney disease (BKD), is difficult to

isolate. Over the last six decades, scientists have utilized a number of bacterial

media for this bacterium such as Minced Chick Embryo medium, Dorset Egg

Medium, Cysteine Blood Agar (KDM1) (Ordal and Earp, 1956), KDM2, which

contains 20 % fetal bovine serum (Evelyn, 1977), Charcoal Agar Medium (Daly

and Stevenson, 1985) and Selective Kidney Disease Medium (SKDM)

supplemented with the four antibiotics; cycloheximide, D-cycloserine, polymyxin

B, and oxolinic acid (Austin et al., 1983). Incubation periods of up to 12 weeks

have been necessary to isolate *R. salmoninarum*.

To shorten the incubation time, Evelyn, et al. (1989) added a nurse *R.*

salmoninarum culture to the center of the KDM2 culture plates. This

modification both accelerated growth of the bacterium and shortened incubation

time. The authors attributed the accelerated growth to the metabolites secreted

by the initial nurse culture. Further, Evelyn et al. (1990) replaced the nurse

culture with 25 µl of filter- sterilized broth that has previously been used to grow

R. salmoninarum (referred to as spent broth) and continued to observe the

accelerated growth. The authors attributed the accelerated growth to the

presence of metabolites produced by the pathogen during its growth. Using the

same approach, Teska (1993) and Starliper et al. (1998) incorporated *R.*

salmoninarum spent medium into the KDM2 medium's constituents (1% v/v,

designated as KDM 2+M) and reported a shortened incubation time and profuse bacterial growth. Most of the previously mentioned studies have been performed on secondary bacterial cultures. However, primary isolation, particularly from carrier fish with negligible tissue bacterial concentration, continues to be a challenge for accurate epizootiological studies (European commission, 1999).

In the State of Michigan, it has been documented that BKD has exist since the early 1950s (Allison, 1958). BKD has been incriminated to cause mass mortalities in the Great Lakes and in hatchery-propagated fish (Holey et al, 1998; Johnson and Hnath, 1991). Despite the potential association between *R. salmoninarum* and clinical cases of BKD in Michigan's salmonines, a relatively limited number of *R. salmoninarum* isolates were retrieved from Michigan and Wisconsin feral spawner salmon in fall of 1991 (Teska, 1994; Starliper 1996). Among the retrieved isolates, those from the Michigan side of Lake Michigan were the most genetically diverse (Starliper, 1996) and of higher virulence (Starliper et al., 1997).

Integral to the host-pathogen interaction of *R. salmoninarum* is the formation of granulomatous host reaction in which bacteria are sequestered (Bruno, 1986; Sami et al., 1992). The presence of such granulomas can impede bacterial isolation using standard bacterial loops and thereby lead to inconsistent isolation results. In this study we combined a modified tissue processing procedure with the use of KDM2+M medium supplemented with antibiotics to select and enhance the primary isolation of *R. salmoninarum*. The

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modified protocol was then used to determine *R. salmoninarum* prevalence

among representative propagated and feral salmonine populations in Michigan.

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MATERIAL AND METHODS

Bacterial growth medium. The medium used throughout this study combined the addition of 10% fetal calf serum recommended by Evelyn et al. (1990) into the KDM2 medium, the four antibiotics recommended by Austin et al. (1983) in the SKDM, and the 1% (v/v) *R. salmoninarum* spent medium used by Teska et al. (1993) and Starliper et al. (1998) in the KDM2+M. The bacterial growth medium used in this study, that combined the modifications listed before, will be referred to as MKDM (modified Kidney Disease Medium). Briefly, MKDM consists of peptone (1 % w/v), yeast extract (0.05 % w/v), L-cysteine HCl (0.1 % w/v) and Cycloheximide (0.005 % w/v) dissolved in distilled water. The medium's pH is adjusted to 6.8. Agar (1.5 % w/v) is added immediately after adjusting the pH of the medium. The medium is sterilized by autoclaving at 121 °C for 15 minutes and left to cool down to 48 °C then the following ingredients are added: new born calf serum (10 % v/v), 0.22 μm filter-sterilized *R. salmoninarum* spent broth (1 % v/v), Oxolinic acid (0.00025 % w/v), Polymyxin B sulfate (0.0025 % w/v) and D-cycloserine (0.00125 % w/v). All MKDM ingredients were purchased from sigma (Sigma Chemical Co, St. Louis, MO, USA) with the exception of agar, which was from Remel (Remel, Lenexa, Kansas, USA).

Fish and sample processing. Details regarding host, age, and geographic origins are found in tables 3-5. In the fall of 2002, a total of 515 feral and captive spawning salmonids were collected from Michigan weirs and state

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fish hatcheries. Fish included 150 returning chinook salmon (*Oncorhynchus tshawytscha*) collected from the Little Manistee River Weir (LMRW), Manistee county, Michigan (Lake Michigan watershed), Swan River Weir (SRW) at Rogers City, Presque Isle county, Michigan (Lake Huron watershed) and Platte River Weir (PRW) at Beulah, Michigan (Lake Michigan watershed). An additional 165 Michigan-adapted coho (*Oncorhynchus kisutch*) and 56 Hinchenbrook coho salmon were collected from the Platte River Weir. Captive brood stock included 60 brook trout (*Salvelinus fontinalis*), 60 lake trout (*Salvelinus namaycush*) that were kept in raceways that receive water from Cherry Creek (Lake Superior watershed) at the Marquette State Fish Hatchery in Michigan's Upper Peninsula. Further, a total of 12 brown trout (*Salmo trutta*) and 12 rainbow trout (*Oncorhynchus mykiss*) were collected from Oden State fish hatchery (OSFH) at Oden, Alanson, Michigan (Lake Michigan watershed). OSFH is the brown and rainbow trout broodstock station and is a major rearing facility for those two species in Michigan. Males and females were equally represented among samples. The sacrifice of the feral spawners entailed exposing the fish to carbon dioxide-laden water, followed by a blow to the head. Following gamete collection, the abdominal cavity was cut open to examine individual internal organs for signs associated with BKD, followed by the collection of kidney tissue samples.

In the spring of 2003, a total of 480 hatchery-reared pre-stocking fingerlings were collected from a number of state fish hatcheries. In brief, a total of 120 brook trout and 120 lake trout fingerlings were collected from MSFH. Additional

180 brown trout and 60 rainbow trout fingerlings were collected from OSFH. In August 2004, a total of 15 brook trout fingerlings were sampled from Cedarbrook Trout Farm at Harrisonville, Michigan.

Cross contamination was avoided by replacing dissecting tools with sterile tools following the dissection of each fish. Attempts to isolate *R. salmoninarum* from kidney tissues were performed by each of the following procedures:

1. streaking a 10 µl loopful of kidney tissue onto MKDM plates,

2. harvesting as much kidney tissue as possible, mincing the tissue in a sterile, plastic Petri dish with scissors, suspending the minced tissue in Hank's balanced salt solution (HBSS, 1:4 w/v, Sigma), and then streaking one hundred microliters (µl) inoculum of the suspension onto MKDM, or

3. transferring the homogenate to 7.5 cm x 18.5 cm Whirl-Pak® bags (Nasco, Fort Atkinson, WI), suspending in HBSS (1:4 w/v), then crushing the suspension in a Biomaster Stomacher-80 (Wolf Laboratories Limited) at the high speed setting for 120 seconds. One hundred µl of the suspension were added to one end of an MKDM plate and then spread over the surface using a sterile bacteriological loop.

Inoculated plates were incubated at 15 °C for up to 30 days and were checked periodically for growth using an inverted dissecting microscope, thus allowing the detection of early colonial growth.

Confirmation of isolates. All colonies were investigated for their conformance with colonial and bacterial morphological criteria of *R. salmoninarum*, as previously detailed in Sander and Fryer (1980) and Austin and Austin (1999). A number of biochemical tests were performed including motility, using motility test medium (DIFCO- BD and Company Sparks, MD, USA), cytochrome oxidase with Pathotec strips (Remel), catalase test with 3 % hydrogen peroxide, hydrolysis of esculin using bile esculin agar (Remel), and DNAse test using DNAse test medium (Remel). Carbohydrate utilization was performed using basal media (DIFCO-BD). The basal medium was prepared according to manufacturer instructions prior to the addition of individual sugars. Ten ml of filter sterilized (0.45 µm) 10 % sugar solution was added to autoclaved and cooled (48 °C) basal media to obtain a final concentration of 1 % with the exception of salicin which was made as 5 % solution to reach 0.5 % final concentration. Each one of the following sugars was added individually to the basal medium to test for the utilization of each sugar: arabinose, glucose, lactose, maltose, rhamnose, salicin, sucrose, sorbitol, xylose. All sugars were from Sigma. Results of biochemical tests were matched against standard *R. salmoninarum* biochemical characters described by Bruno and Munro (1986).

Nested PCR. Single bacterial colonies were identified using highly specific oligonucleotide primers designed by Pascho et al. (1998), which amplify a region of the gene encoding the *R. salmoninarum* p57 antigen in a nested polymerase chain reaction (nPCR). The nPCR assay using these primers is

considered the method of choice to confirm *R. salmoninarum* isolates (OIE 2003, Pascho and Elliott, 2004). Briefly, DNA was extracted from each isolate using DNeasy Extraction Tissue Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol with minor modifications. Bacterial pellets were obtained by centrifugation at 6000 *g* for 20 minutes at 4 °C and subsequently incubated with lysozyme buffer that consisted of 180 μl of 20 mg lysozyme (Sigma) , 20 mM Tris-HCl, pH 8.0; 2 mM EDTA (Sigma) and 1.2 % (v/v) Triton X100 (Sigma) at 37 °C for 1 hour. The nPCR method and the primers recommended by Pascho et al. (1998) were used with slight modification to the volume of utilized DNA (5 μl for first round and 2 μl for second round PCR reaction), water, and master mixes (45 μl for first round and 48 μl for second round nPCR reaction). The controls were composed of a PCR mixture containing no DNA template (reagent negative control), a positive *R. salmoninarum*, and a positive tissue control. A volume of 10 μl of the nPCR product and controls were mixed with 2 μl of 6X loading dye (Sigma) and used to load a gel consists of 2 % agarose (Invitrogen Life Technologies, Carlsbad, CA). Each electrophoresis gel included a 1 k bp (with 100 bp increments) DNA ladder (Invitrogen). Gels were run at 100 volt for 30 minutes in 1 X Tris Acetate Buffer (1 X TAE) (Sigma). Gels were visualized with the KODAK EDAS Camera System and UV Trans-illuminator. Suspect colonies were considered positive for *R. salmoninarum* when a 320 bp band was detected.

Antibiogram. Two media were used to test the isolates for sensitivity to antibiotics using a modified Kirby-Bauer disc diffusion method (Bauer et al., 1966):

1. Antibiotic free MKDM agar medium

2. Modified Muller's Hinton agar medium (MMHA): Muller's Hinton Medium (MHA) is used in standard antibiotic sensitivity testing. L-cysteine was added to MHA to support the growth of *R. salmoninarum*. The MMHA was prepared by adding 1 g L-cysteine HCl (Sigma) to 0.5 g yeast extract (Sigma), 100 ml fetal bovine serum (Sigma), and 38 g of dehydrated MH agar medium (Remel), and then suspending the mixture in double distilled water to obtain a 1000 ml media volume.

The antibiogram was performed on 14 representative *R. salmoninarum* isolates. Five-day-old colonies were suspended in sterile saline to obtain turbidity equivalent to a 0.5 McFarland standards (Remel). From each bacterial suspension 200 µl was spread onto antibiotic free MKDM and MMHA plates (one plate each/isolate). Cultures were left for a few minutes to allow the bacteria to adsorb to the agar surface. Using an automatic dispenser (Remel), antibiotic discs (5 mm in diameter) were placed on the culture plate surface. The plates were then inverted and incubated at 15°C for 5 days in a subambient temperature incubator (Fisher Scientific Company L.L.C. Hanover Park, IL). Culture plates were observed and results recorded by measuring the diameter of the zone of inhibition in millimeters around each disc using a calibrated ruler.

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The following antibiotic discs (all from Remel) were used in the

antibiogram: chloramphenicol (C), terramycin (TE), sulmethoxazole -

trimethoprim (SXT), carbenicillin (CAR), erythromycin (E), azithromycin (AZM),

Kanamycin (K), clindamycin (DA), polymyxin B sulfate (PB), novobiocin (NV),

ofloxacin (OFX), ciprofloxacin (CIP), enrofloxacin (ENO) and norfloxacin (NOR).

Preservation and storage of isolates. Identified *R. salmoninarum*

isolates were cryoperserved and deposited at the Aquatic Animal Health

Laboratory, Michigan State University. Bacterial suspensions were prepared

from 5 day-old cultures in MKDM broth (not supplemented with antibiotics) and

then stored at –80 °C.

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RESULTS

Effects of sample processing and culture technique on primary isolation of *Renibacterium salmoninarum*. When MKDM was streaked with a loopful (10 μl) of infected kidney tissue, colonies were evident 15-20 days post incubation. Using minced kidney of the same tissue and increasing the inoculum to 100 μl shortened the incubation time to 10-15 days. However, when kidney tissue samples were stomached for 120 seconds, profuse growth was achieved within a relatively short period (5-10 days). *Renibacterium salmoninarum* colonies grew on and around streaked tissues and were creamy, glistening, smooth, convex and 1-2 mm in diameter. Representative colonies were individually picked and their identities confirmed using nested PCR assay. Because of the astounding success in shortening the incubation period and the absence of contaminants, stomaching of minced tissues and inoculating MKDM plates with 100 μl of tissue homogenate became the routine tissue processing procedure for the primary isolation of *R. salmoninarum* from tissues in this and subsequent studies.

Prevalence of *R. salmoninarum* isolated from Michigan salmonines
1. Feral salmon population. Tables (3-5) show the numbers of fish examined, prevalence, and numbers of fish exhibiting BKD clinical signs. Most of the examined fish looked healthy and exhibited healthy-looking kidneys and internal organs. A number of fish in each of the species and lots examined

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displayed whitish abscesses like nodules that sometimes coalesce to form patches in the kidneys' parenchyma. The prevalence of the pathogen was highest in chinook salmon from the Platte River Weir chinook (96.3%), followed by Little Manistee weir chinook (82.5%), and Swan River weir chinook (48.3%)(table 3). As shown in figure 1 and 2, prevalence seems not to differ among males and females. Coho salmon spawners showed prevalence rates that reached up to 100% in the Hinchenbrook strain, with more than half of the examined fish showing clinical BKD signs (table 3). As depicted in Figure (2), BKD lesions were more apparent in females coho salmon than males.

2. Prevalence in captive broodstocks. Results indicated that *R. salmoninarum* is also widespread among captive broodstocks (table 4), with brown trout (the Wild Rose strain) being the highest in both prevalence and percent of clinical cases, followed by rainbow trout, brook trout, and Lake trout stocks.

3. Prevalence in propagated offspring fish. The prevalence of *R. salmoninarum* in propagated Iron River Brook trout fingerlings were remarkably high (83 %), with 50 % of the fish presenting with lesions typical of BKD. In addition, propagated Assinica BKT fingerlings also showed relatively high *R. salmoninarum* prevalence (42 %), with 16.66% showing clinical signs. Similarly, the *R. salmoninarum* prevalence and percentage of fish (60 %) with clinical signs were very high in BKT obtained from a fish farm (table 5).

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Fingerlings of lake trout, three strains of brown trout, and rainbow trout showed relatively lower prevalences (<25%) and lesser numbers of fish with clinical signs (table 5).

Confirmation of the retrieved *R. salmoninarum* isolates. A total of 566 *R. salmoninarum* isolates were retrieved from infected fish tissues over a two-year period. All colonies were creamy-whitish, glistening, 1-2 mm in diameter, rounded, and smooth. Old colonies (over 40 days incubation) showed granular white or crystalline appearance. Gram staining demonstrated Gram-positive diplo- or coccobacilli. No capsules, metachromatic granules, or bipolarity were detected in the stained slides of all isolates.

I. Molecular Confirmation. Nested PCR performed on all isolates exhibited the *R. salmoninarum* characteristic 320 bp band.

II. Biochemical Reactions. Results of the conventional biochemical testing of 12 representative isolates demonstrated isolate uniformity. Isolates were non-motile, catalase positive, cytochrome oxidase negative, esculin hydrolysis negative, DNAse negative and carbohydrate utilization negative (table 6).

III. Antibiogram of the *R. salmoninarum* Isolates. Tables 7 and 8 show the details of the *R. salmoninarum* antibiogram results. Results indicate that, after 5-10 days incubation, the inhibition zones obtained from isolates cultured on antibiotic-free MKDM medium were sharper and more obvious than those

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obtained by culture on MMHA medium. However, both media yielded relatively similar results. All 12 isolates were highly sensitive to enrofloxacin and ciprofloxacin with a 27mm and 23 mm average inhibition zone diameters respectively). Ten isolates were markedly sensitive to terramycin (21 mm average inhibition zone). Interestingly, two of the isolates retrieved from captive brown trout broodstock were resistant to erythromycin and azithromycin, while the remaining 10 isolates were sensitive or intermediately sensitive to erythromycin, azithromycin and sulfamethoxazole –trimethoprim, with average inhibition zones of 17mm, 15.5mm and 18mm, respectively. Moreover, all isolates were resistant to polymyxin B and clindamycin (average inhibition zone diameter of 0, 4mm respectively). In addition, most of the isolates were resistant to kanamycin; with an average inhibition zone diameter of 9mm. Isolates were sensitive to chloramphenicol, novobiocin, and carbenicillin showing average inhibition zones diameters of 20 mm, 14mm and 16mm respectively.

DISCUSSION

Results from this study demonstrated that the combined use of MKDM medium and stomaching minced tissues diluted in HBSS was optimal for primary isolation of *R. salmoninarum*. The antibiotics Austin et al. (1983) incorporated into the medium combined with a shorter incubation period minimized the growth of contaminating bacteria and fungi. There are a number of factors that may have led to the improved growth of *R. salmoninarum* that was observed. First, thorough mincing and homogenization may have caused release of *R. salmoninarum* from granulomas and fibrous layers. Second, the relatively aggressive tissue processing may have led to a massive release of bacterial metabolites that are known to boost bacterial growth *in vitro* (Evelyn et al.1989; 1990). Third, a major part of the kidneys (posterior and anterior) were used in homogenization, a matter that increases the likelihood of isolating bacteria even if present in low numbers, as in the case of carrier fish. Pascho et al. (1987) were able to double the likelihood of isolating *R. salmoninarum* from infected fish by combining samples taken from three different spots in the kidneys of individually tested fish. Fourth, a heavy tissue inoculum (100 μl) was used, a matter which enhances the likelihood of bacterial isolation when compared to using a loopful (~ 10 μl only). Fifth, mixing kidney tissues with four times their weight of HBSS may have diluted inhibitory molecules present in tissue that Evelyn et al. (1981); Daly and Stevenson 1988 and Olsen et al. (1992) believed to lower the likelihood of isolating *R. salmoninarum* from homogenized kidney tissues. Last, the unique formula of HBSS with its rich

inorganic ions and electrolyte content might have played a role in adjusting the pH and osmotic balance. Our improved *R. salmoninarum* growth may be a result of an additive effect from some or all of the aforementioned points. Findings of the current study clearly demonstrate the advantages of this modified tissue processing in the improvement of primary isolation of *R. salmoninarum* from infected tissues, even if infection intensity is relatively low. Conventional biochemical tests revealed that *R. salmoninarum* isolates tested in this study were non-motile, catalase positive, cytochrome oxidase negative, esculin hydrolysis negative, DNAse negative and carbohydrate utilization negative. These findings coincide with the standard biochemical criteria of *R. salmoninarum* described by Smith (1964); Sanders and Fryer (1980); Bruno and Munro (1986) and indicate the biochemical uniformity of Michigan isolates among themselves and among other *R. salmoninarum* strains isolated worldwide. Our identifications were further confirmed with nPCR results as all *R. salmoninarum* isolates had the 320 bp band characteristic for *R. salmoninarum* (Pascho et al., 1998).

The antibiogram performed on 12 *R. salmoninarum* representative isolates indicated that all tested isolates were highly sensitive to enrofloxacin, which is in agreement with the report of Hsu et al. (1994). In the study of Hsu et al. (1994), the authors performed an *in vitro* sensitivity test using the standard MIC (minimal inhibition concentration) method and an *in vivo* efficacy trial using enrofloxacin medicated food to treat juvenile rainbow trout experimentally infected with *R. salmoninarum*. The authors concluded that *R. salmoninarum* is

highly sensitive to enrofloxacin and suggested that the low MICs, high

bioavailability, and large volume distribution of enrofloxacin make it a good

candidate for treatment of BKD. Results of the present study showed that all

isolates were highly sensitive to ciprofloxacin and intermediate sensitive to

ofloxacin and norfloxacin. Moreover, the sensitivity of the isolates to

terramycin, chloramphenicol, novobiocin, sulfamethaxozole - trimethoprim,

carbenicillin and the resistance to polymyxin B coincide with previous reports

(Austin, 1985; Wolf and Dunbar, 1959; Goodfellow et al., 1985).

An unexpected result was the presence of two erythromycin and azithromycin

resistant isolates (Oden BNT-BS-02 and Oden BNT-BS1-02), since the "wild –

type" *R. salmoninarum* strains are known to be sensitive to macrolide

antibiotics (Austin, 1985). However, this finding corroborates with Bell et al.

(1988), who were able to develop erythromycin-resistant *R. salmoninarum*

strains *in vitro* but were unable to describe the mechanism behind this

resistance. The brown trout stock from which these two isolates were retrieved

has never been treated with erythromycin. Resistance to erythromycin and

other macrolide antibiotics is believed to be associated with mutational (Tanaka

et al., 1968) or plasmid –mediated blockage (e.g. methylation) (Weisblum et al.,

1971) of the binding sites on the 23 S RNA. Whether erythromycin-resistance

observed in the brown trout strain has developed as a result of any of the

mechanisms described for other Gram-positive bacteria is currently unknown

and deserves further investigation.

From the relatively limited survey performed in this study, a number of interesting results were demonstrated that would vastly improve our current understandings of the status of BKD in Michigan, as well as help shape the design of future epidemiological studies. First, it is quite clear that *R. salmoninarum* is widespread in Michigan, as it has been isolated from every fish population and lot that was tested (tables 3-5). Second, infected fish are not only carriers, but some also developed clinical kidney lesions consistent with BKD. Third, vertical transmission seems to play a major role in BKD transmission in brown and rainbow trout, as fingerlings were kept throughout their life in raceways supplied with well water. Fourth, results also suggested that *R. salmoninarum* prevalence varies among the species examined, with brook trout and coho salmon being the highest in both prevalence and presence of kidney lesions. Fifth, even within the same species, fish strain seems to play a role in susceptibility to *R. salmoninarum*. For example, the Hinchenbrook coho salmon strain showed higher prevalence than that of the Michigan adapted coho salmon strain collected from Platte River. Also, the Hinchenbrook coho salmon strain showed relatively higher percent of kidney lesions when compared to Michigan-adapted coho salmon strain. This increased susceptibility could be due to the fact that the Hinchenbrook coho salmon strain was recently introduced to Michigan (in the 1990s), while the other coho salmon strain has adapted to the Great Lakes basin since it's introduction in the 1960s (Michigan Department of Natural Resources (MDNR). This also applies for the brook trout Iron River strain, which has recently been

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adopted for propagation purposes, as opposed to the Assinica strain, which has been domesticated for five decades. On the contrary, there have been no noticeable differences in *R. salmoninarum* prevalence among three strains of brown trout. Variation in resistance to BKD among strains within the same species has also been reported by Winter et al. (1980) who made a similar observation with steelhead trout (*Oncorhynchus mykiss*). Last, in the case of chinook salmon, results suggest that *R. salmoninarum* prevalence varied among sites to which spawning runs return. For example, adult Platte River chinook salmon coming from Lake Michigan showed higher *R. salmoninarum* prevalence (96 %) and higher percent of clinical cases (11 %) when compared to Swan River chinook salmon (48 %, 5 % respectively) coming from Lake Huron. The same trend was observed in chinook salmon from the Little Manistee Weir, which exhibited a higher *R. salmoninarum* prevalence than those found at the Swan River weir. Spatial distribution and population density could play a role in elevating the prevalence of BKD among fish populations (Reno, 1998).

In conclusion, this study has shed light on the widespread *R. salmoninarum* infections occurring in Michigan's salmonids. The tissue processing protocol, combined with the modified medium, has facilitated the isolation of several hundred *R. salmoninarum* isolates. These isolates constitute an important resource for scientists studying *R. salmoninarum* and BKD in the United States in general and the Great Lakes in particular.

Host	Weir	# of fish tested	Prevalence (positive/total)		
			Apparently healthy fish	Fish with gross kidney Lesions *	Total
Chinook salmon	Little Manistee River	63	47/ 63 (74.60 %)	5/63 (7.93 %)	52/63 (82.53 %)
	Swan River	60	26/60 (43.33 %)	3/60 (5 %)	29/60 (48.33 %)
	Platte River	27	23/27 (85.18 %)	3/27 (11.11 %)	26/27 (96.29 %)
Michigan - adapted coho salmon	Platte River	165	112/165 (67.87 %)	30/165 (18.18 %)	142/165 (86 %)
Hinchenbrook coho salmon		56	26/56 (46.42 %)	30/56 (53.57 %)	56/56 (100 %)
Total		371	234/371 (63 %)	71/371 (19.1%)	305/371 (82.2 %)

Table 3. Prevalence of *Renibacterium salmoninarum* in fall returning chinook and coho salmon spawners from Lake Michigan and Lake Huron watersheds.

* White-creamy patches in kidneys

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Host	Number of fish examined	Prevalence		
		Apparently healthy	Fish with kidney lesions	Total
Brook trout	60	22 (36.66 %)	10 (16.66 %)	32 (53.33 %)
Lake Trout	60	40 (66.66 %)	3 (5 %)	43 (71.66 %)
Brown Trout	12	8 (66.66 %)	4 (33.33 %)	12 (100 %)
Rainbow trout	12	9 (75 %)	2 (16.66 %)	11 (91.66 %)
Total	144	79/144 (54.9%)	19/144 (13.2%)	98/144 (68%)

Table 4. Prevalence of *Renibacterium salmoninarum* in captive char and trout broodstocks collected in the fall 2002 from Michigan state fish hatcheries.

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Fish Species	Number of fish tested	Prevalence		
		Apparently healthy	Fish with kidney lesion *	Total
Brook trout Assinica	60	15 (25 %)	10 (16.7 %)	25 (41.7 %)
Brook trout Iron River	60	20 (33.3 %)	30 (50 %)	50 (83.3 %)
Brook trout Cedarbrook	15	0 (0 %)	9 (60 %)	9 (60 %)
Lake Trout	120	22 (18.3 %)	0 (0 %)	22 (18.3 %)
Brown trout (Wild Rose)	60	8 (13.3 %)	2 (3.3 %)	10 (16.7 %)
Brown trout Seeforellen	60	14 (23.3 %)	1 (1.7 %)	15 (25 %)
Brown trout Gilchrist	60	12 (20 %)	3 (5 %)	15 (25 %)
Rainbow trout	60	8 (13.3 %)	2 (3.3 %)	10 (16.7 %)
Total	495	99/495 (20%)	57/495 (11.5 %)	156/495 (31.5 %)

Table 5. Prevalence of *Renibacterium salmoninarum* in 10-15 month-old propagated fish. All samples were collected in January 2003 except those from Cedarbrook, which were collected in August 2004.

Isolate ID	Conventional biochemical tests					Motility
	C	O	E	D	CHO	
Marquette BKT-BS-02	+	-	-	-	-	-
Marquette LT-02-A	+	-	-	-	-	-
Wild Cherry Creek BKT44-02	+	-	-	-	-	-
Oden BNT-BS-02	+	-	-	-	-	-
Marquette LT-02-B	+	-	-	-	-	-
Oden RBT-02	+	-	-	-	-	-
Marquette LT 24-02	+	-	-	-	-	-
Platte River CH-02	+	-	-	-	-	-
Oden BNT-BS1-02	+	-	-	-	-	-
Platte River HB-02	+	-	-	-	-	-
Swan River CH5-02	+	-	-	-	-	-
Platte River CO-02	+	-	-	-	-	-

Table 6. Results of conventional biochemical and motility tests performed on the 12 representative *Renibacterium salmoninarum* isolates.
Conventional biochemical tests used in this study are catalase (C), oxidase (O), esculin hydrolysis (E), DNAse (D), and carbohydrate utilization tests (CHO).

Antibiotic		Inhibition zone diameter standards in mm			
Name	Code	Disk potency	Resistant	Intermediate	Sensitive
Terramycin	TE	30 µg	No zone	<15	≥15
Chloramphenicol	C	30 µg	No zone	13-17	≥18
Trimethoprim - Sulfamethoxazole	SXT	25 µg	No zone	<15	≥15
Erythromycin	E	15 µg	No zone	<15	≥15
Azithromycin	AZM	15 µg	No zone	<15	≥15
Clindamycin	DA	2 µg	No zone	<15	≥15
Ciprofloxacin	CIP	5 µg	No zone	<15	≥15
Enrofloxacin	ENO	5 µg	No zone	<15	≥15
Ofloxacin	OFX	5 µg	No zone	13-15	≥16
Norfloxacin	NOR	10 µg	No zone	14-16	≥17
Kanamycin	K	30 µg	No zone	14-17	≥18
Carbenicillin	CAR	100 µg	No zone	<15	≥17
Novobiocin	NV	30 µg	No zone	<10	≥10
Polymyxin B Sulfate	PB	300 U	No zone	9-11	≥12

Table 7. Standard concentration and expected inhibition zones for each used antibiotic disc.

Isolate ID / Antibiotic	1	2	3	4	5	6	7	8	9	10	11	12	Mean	SE	SD
TE	25	20	20	8	25	27	25	24	8	26	25	22	21.25	1.89	6.50
SXT	20	18	19	18	20	22	18	14	12	18	19	18	18.00	0.76	2.70
C	25	22	12	20	24	18	17	20	20	27	18	16	20.00	1.20	4.16
CAR	17	18	7	18	15	14	12	14	10	18	18	8	14.00	1.16	4.03
E	19	22	13	7	10	20	20	23	9	20	17	20	16.70	1.57	5.46
AZM	17	20	12	8	12	17	18	22	8	17	19	16	15.50	1.30	4.52
K	10	8	12	10	9	14	10	10	6	8	10	6	9.40	0.65	2.27
DA	6	0	0	0	8	10	0	6	0	6	8	0	3.66	1.15	3.98
PB	0	0	0	0	0	0	0	0	0	0	0	0	0.00	0.00	0.00
NV	15	20	10	14	17	14	13	15	17	18	18	16	15.60	0.77	2.67
OFX	20	18	20	19	20	20	19	18	16	20	19	18	19.00	0.35	1.24
CIP	27	23	22	25	22	28	20	19	17	22	22	25	22.70	0.92	3.20
ENO	30	32	28	27	31	32	25	18	19	28	30	29	27.40	1.33	4.64
NOR	19	20	18	16	18	20	17	15	16	18	19	20	18.00	0.49	1.70

Table 8. Inhibition zones in millimeters (mm) produced by the 12

***Renibacterium salmoninarum* isolates used in the antibiogram.**

Note: 1 (Marquette BKT-BS-02), 2 (Marquette LT-02-A), 3 (Cherry Creek BKT44-02), 4 (Oden BNT-BS-02), 5 (Marquette LT-02-B), 6 (Oden RBT-02), 7 (Marquette LT 24-02), 8 (Platte River CH-02), 9 (Oden BNT-BS1-02), 10 (Platte River HB-02), 11 (Swan River CH5-02), 12(Platte River CO-02)
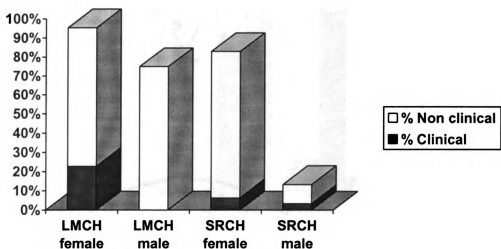
Figure 1. Prevalence of *Renibacterium salmoninarum* in male and female returning chinook salmon spawners from Lake Michigan and Lake Huron watersheds. Prevalence was determined by calculating % positive *Renibacterium salmoninarum* culture retrieved from fish with clinical or without clinical lesions.

* LMCH: Little Manistee River Weir chinook salmon
* SRCH: Swan River Weir chinook salmon.
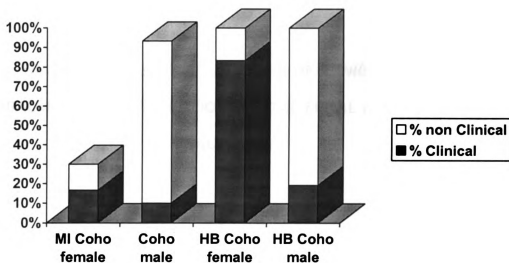
Figure 2. Prevalence of *Renibacterium salmoninarum* in male and female returning coho salmon spawners from Platte River Weir, Lake Michigan watershed. Prevalence was determined by calculating % positive *Renibacterium salmoninarum* culture retrieved from fish with clinical or without clinical lesions.

* MI Coho: Platte River Weir Michigan adapted coho salmon
* HB Coho: Platte River Weir Hinchenbrook coho salmon.

CHAPTER THREE

DIAGNSOTIC TESTING PATTERNS OF *RENIBACTERIUM* *SALMONINARUM* INFECTION IN SOME FERAL AND CAPTIVE SALMONINES

ABSTRACT

Bacterial kidney disease, caused by *Renibacterium salmoninarum (R. salmoninarum)*, is a slowly progressing disease that hampers salmon conservation and restoration programs in North America. The purpose of this study was to track the progress of naturally occurring *R. salmoninarum* infection in some of Michigan's salmonid stocks using nested polymerase chain reaction (nPCR), quantitative enzyme linked immunosorbent assay (Q-ELISA), and culture. The Q-ELISA test detected 67.6 % infection prevalence, which is lower than culture (77.2 %) or nPCR (94.2 %), yet it provided semi-quantitative data on infection intensity. The disagreement in results among the three assays may reflect the different phases of *R. salmoninarum* infection at the time of sampling. The testing results demonstrated the presence of six patterns, with each of the patterns representing a probable stage along the course of *R. salmoninarum infection.*

Findings also suggested that fish stocks tested in this study are not uniform

in the distribution of the diagnostic patterns.

INTRODUCTION

Bacterial Kidney Disease (BKD) of salmonines, caused by

Renibacterium salmoninarum, is a slowly progressing systemic infection,

which often causes high losses among infected fish (Fryer and Sanders

1981). In addition to horizontal transmission, *R. salmoninarum* can be

transmitted vertically (Evelyn et al. 1986 a,b). Therefore, BKD is a major

concern for salmonine conservation and restoration programs worldwide in

general, and in the Great Lakes basin, in particular (Faisal and Hnath 2005).

Since the first report of Kidney Disease in 1930, a number of diagnostic

assays have been developed to determine the presence of *R. salmoninarum*

in infected fish tissues. Culture on selective media, fluorescent antibody

technique, quantitative ELISA (Q-ELISA), and nested polymerase chain

reaction (nPCR) are currently the most common diagnostic techniques used

in the detection of *R. salmoninarum* (Pascho and Elliott 2004; OIE 2003).

When multiple diagnostic tests were performed on the same sample,

numerous discrepancies among findings were observed (Cipriano et al.

1985; Sakai et al. 1989; White et al. 1995; Jansson et al. 1996; Miriam et al.

1997; Pascho et al. 1998). While these inconsistencies were often difficult

to interpret, most authors concentrated on these discrepancies to merely

evaluate the sensitivities of various detection assays (Jansson et al. 1996;

Chase and Pascho 1998; Pascho et al. 1998). For example, it has been

estimated that the nested PCR assay, developed by Chase and Pascho

(1998), which uses primers specific for conserved regions of the major

soluble antigen (msa) gene, has enabled the detection of as little as 10

bacterial cells/gram of kidney tissue (Pascho et al. 1998). Culture, on the

other hand, requires the presence of 40-100 bacterial cells per gram tissue to

ensure bacterial isolation (Lee 1989; Miriam et al. 1997). The quantitative

ELISA assay primarily targets bacteria-secreted soluble proteins (Pascho

and Mulcahy 1987; Meyers et al. 1993; Pascho et al. 1998). It has been

estimated that Q-ELISA requires a minimal bacterial concentration of 1.3X

10^4 bacterial cells/ml of ovarian fluid and 10^3-cells/g kidney tissues are

needed to produce consistent positive results (Pascho et al. 1998).

Mechanisms involved in the initiation of infection, progression of the

disease, death, and/or recovery from natural *R. salmoninarum* infections are

currently unknown (reviewed in Evenden et al. 1993; Wiens and Kaattari

1999). It is well documented that *R. salmoninarum* can be transmitted

vertically from parents to offspring (Evelyn et al. 1984; 1986). Horizontal

transmission is believed to occur primarily through the oral-fecal route (Balfry

et al. 1996), and to some extent through the gills (Flaño et al. 1996) and skin

lesions (Evenden et al. 1993). *Renibacterium salmoninarum* infections can

persist in fish lacking clinical signs, while in others, the infection may

progress, causing clinical signs with bacterial numbers reaching up to 10^9

colony forming units/ g of kidney tissues prior to death (Bruno 1986; Evelyn

et al. 1996).

Renibacterium salmoninarum secretes a number of soluble proteins that seem to play a role in pathogenicity (Austin and Rodgers 1980; Bruno and Munro 1986; Wiens and Kaattari 1991; Hamel 2001). These bacterial proteins form complexes with fish antibodies that are deposited in kidney glomeruli and are then slowly eliminated (Sami et al. 1992). Therefore, the aim of this study is to utilize the limits of detection for each assay, what each assay detects, and the collective results of these assays to shed some light on the kinetics and course of infection within certain fish population.

MATERIALS AND METHODS

Fish. Between October-November 2002, a total of 364 feral and captive

spawning salmonids were collected from Michigan weirs and state fish

hatcheries. Fish included 100 returning chinook salmon (*Oncorhynchus*

tshawytscha) collected from the Little Manistee River Weir (LMRW),

Manistee county, Michigan (Lake Michigan watershed) and Swan River Weir

(SRW) at Rogers City, Presque Isle county, Michigan (Lake Huron

watershed). An additional 131 Michigan-adapted coho (*Oncorhynchus*

kisutch) and 53 Hinchenbrook coho salmon were collected from the Platte

River Weir (PRW) at Beulah, Michigan (Lake Michigan watershed). Captive

brood stock included 41 brook trout (*Salvelinus fontinalis*) and 39 lake trout

(*Salvelinus namaycush*). The captive stocks were kept in raceways that

receive water from Cherry Creek (Lake Superior watershed) at the Marquette

State Fish Hatchery in Michigan's Upper Peninsula. Males and females were

equally represented among samples. The sacrifice of the feral spawners

entailed exposing the fish to carbon dioxide-laden water, followed by a blow

to the head. Following gamete collection, the abdominal cavity was cut open

to examine individual internal organs for signs associated with BKD, followed

by the collection of approximately one gram of tissue from anterior, posterior

and middle kidney sections. Cross contamination was avoided by replacing

dissecting tools with sterile tools following the dissection of each fish.

Sampling and sample processing: Samples from fish were analyzed individually unless otherwise indicated. Kidney samples representing the anterior, posterior, and middle sections of the kidney were transferred in sterile 7.5 cm x 18.5 cm Whirl Pak® bags (Nasco, Forte Atkinson, and WI), kept on ice, and were softened as much as possible through multiple cycles of physical pressure. The homogenized kidney tissues were diluted in 1:4 (w/v) Hank's Balanced Salt Solution (HBSS, Sigma Chemical Co, St. Louis, MO, USA) and then stomached for 2 minutes at high-speed using the Biomaster Stomacher-80 (Wolf Laboratories Limited, Pocklington, York, UK). In the case of coelomic fluid, 1 ml of each sample was diluted 1:2 (v/v) in HBSS for Q-ELISA testing. In the case of the milt, 1ml from each sample was diluted 1:2 (v/v) in HBSS for Q-ELISA testing.

Culture. Isolation of *R. salmoninarum* was performed as described in Chapter 2 in this thesis. Briefly, 100 µl aliquots of stomached kidney tissues were spread onto MKDM (Eissa 2005, chapter 2). Culture plates were incubated for a period of 20 days at 15 °C. Inoculated plates were checked every 5 days for bacterial growth. Identification of the isolates was done according to the standard morphological criteria of *R. salmoninarum* (Sanders and Fryer 1980; Austin and Austin 1999). Molecular confirmation of the isolates was done using the nPCR method described by Pascho et al. (1998).

Measurements of *Renibacterium salmoninarum* antigen using the Quantitative Enzyme-linked Immunosorbent Assay (Q-ELISA). Aliquots (250 µl) of each sample were transferred into 1.5 ml safe lock microfuge tubes, to which an equal volume of 0.01 M Phospate Buffered Saline Tween 20 (0.05 %) (PBS-T20) (Sigma) with 5 % natural goat serum (Sigma) (Olea et al. 1993) and 50 µl CitriSolv solution (Fisher Chemicals, Fairlawn, New Jersey, USA) (Gudmunsdottir et al. 1993) were added. The solution was then thoroughly mixed via vortexing, incubated at 100 °C on heat blocks with a rotary shaker for 15 minutes, followed by 2 hours of incubation at 4 °C. After incubation, the mixture was centrifuged at 6000g for 15 minutes at 4 °C. The aqueous supernatant of each sample was carefully collected and then transferred to a 1.5 ml microfuge tube for Q-ELISA testing. The Q-ELISA method used in this study is that described in detail by Pascho and Mulcahy (1987) as well as Alcorn and Pascho (2000). The positive-negative threshold absorbances were calculated according to the method described by Meyers et al. (1993) .The positive–negative cutoff absorbance for the kidney homogenate was 0.10. The tested positive samples were assigned the following antigen level categories: low (0.10 to 0.19), medium (0.20-0.99) and high (1.000 or more) (Pascho et al. 1998).

Nested PCR. Bacterial DNA was extracted using the DNeasy tissue extraction kit (Qiagen-Valencia, CA, USA). DNA was extracted from 100 µl aliquots of kidney tissue homogenates according to manufacturer's

instructions and the method described by Pascho et al. (1998) with minor modifications. The tissue pellets were obtained by centrifugation at 6000 g for 20 minutes at 4 °C and then incubated with lysozyme buffer consisting of 180 µl of 20 mg lysozyme (Sigma-Aldrich, Inc. St. Louis, MO), 20mM Tris-HCl (pH 8.0), 2 mM EDTA (Sigma) and 1.2 % (v/v) Triton X 100 (Sigma) at 37 °C for 1 hour. The nPCR method used primers recommended by Pascho et al. (1998) with slight modifications to the volume of DNA (5µl for the first round and 2µl for the second round nPCR) and master mixes (45µl for the first round and 48µl for the second round nPCR). The controls were composed of a PCR mixture containing no DNA template (reagent negative control), positive *R. salmoninarum* and positive tissue control. A volume of 10 µl of the nPCR product and controls were mixed with 2 µl of 6X loading dye (Sigma) and used on a 2 % agarose gel (Invitrogen Life Technologies, Carlsbad, CA). Each electrophoresis gel included a 1kbp DNA ladder with 100 bp increments (Invitrogen). Gels were run in 1 X Tris Acetate (1 X TAE) Buffer (Sigma). Gels were visualized under the KODAK EDAS Camera System and UV Trans-illuminator. Samples were considered positive when a 320 bp band was detected.

RESULTS

Among the 364 fish examined, 343 fish (94.2%) were positive in the nPCR assay, 281 (77.2%) were culture positive and 246 (67.6%) were positive with the Q-ELISA. Over half (53.3%) of the fish used in this study gave positive results by all three diagnostic assays (table 9). The consistency among findings was highest in the case of Hinchenbrook coho (81.1%), followed by brook trout (63.4%). Only 7 fish (1.9%) were negative by all three diagnostic assays. As displayed in table (9), most Q-ELISA positive fish possessed low *R. salmoninarum* antigen concentrations (193/231, 83.5%), the highest proportions of which were in the LMRW Manistee chinook salmon and PRW Michigan-adapted coho salmon (64.3 %, 67.9 % respectively). Fish with medium and high *R. salmoninarum* antigen concentrations were found primarily in Hinchenbrook coho salmon (45.2%) and brook trout (29.2%) stocks.

Combining the results of the three diagnostic assays performed on the same samples, 6 patterns were recognized. Pattern 1 represents fish that were positive with the nPCR only (24/364, 6.7%). Pattern 2 represents fish positive with both nPCR and culture assays (87/364, 23.9%). The majority of fish (194/364, 53.3%) were in Pattern 3, with positive results in all three diagnostic techniques. Pattern 4 represents fish positive with nPCR and Q-ELISA (38/364, 10.4%). Pattern 5 represents fish that were barely positive in the Q-ELISA assay (14/364, 3.8%). Pattern 6 was the least represented,

with only 7 fish (1.9%) that had negative results with all three assays used in this study.

The distribution of fish representing each of the patterns differed among the fish stocks tested. For example, the majority of LMRW chinook salmon were in patterns 2 and 3 (Figure 3A), while SRW chinook salmon showed a wider distribution consisting of over half of the fish in patterns 1 and 2 (Figure 3B). In the case of PRW Michigan-adapted coho, almost all fish were in patterns 2-4 (Figure 4A). On the other hand, 75% of the Hinchenbrook coho salmon strain belonged to pattern 3 (Figure 4B). Similarly, the two captive *Salvelinus* spp. broodstocks, though kept in the same hatchery, exhibited different diagnostic testing patterns (Figures 5 - 6), with over 60% of the brook trout belonging to Pattern 3.

DISCUSSION

Findings clearly suggested that *R. salmoninarum* infection is widespread in adult fish of the stocks tested in this study. Only 7 fish were negative out of 364 when tested by the three diagnostic assays. While these figures are staggering, one should not be surprised since *R. salmoninarum* has existed in Michigan salmonines for at least 50 years (Allison 1958) and was involved in the massive chinook salmon die offs of the 1980s in Lake Michigan (Johnson and Hnath 1991; Holey et al. 1998). However, it should also be emphasized that fish tested in this study were spawning adults (>4 years old); a matter that increases the likelihood of exposure to *R. salmoninarum* and allows time for the slow progression of this infection. Moreover, sampling took place at the peak of the spawning season, meaning that the fish were subjected to multiple stressors, such as starvation, hormonal changes, and physical pressure on internal organs due to distension of the gonads. Therefore, infection rates obtained in this study should not be considered representative of the overall *R. salmoninarum* prevalence at the population level. Regardless of these factors, data strongly suggested that *R. salmoninarum* continues to be enzootic in Michigan's salmon and char species.

In other areas of the world where *R. salmoninarum* is enzootic, prevalence of infection in feral and wild fish species have reached up to 100%. For example, in Iceland, arctic char and brown trout reached infection

levels of 100% and 81%, respectively (Jónsdóttir et al. 1998). In North

America, *R. salmoninarum* infection rates of 83% in brook trout from

Wyoming, USA (Mitchum et al. 1979), and 35% in returning Atlantic salmon

in the Margaree River, Halifax, Canada (Paterson et al. 1979) were reported.

The findings also suggested that nPCR performed with primers targeting

the *msa* gene is superior to culture and Q-ELISA methods in detecting *R.*

salmoninarum infection. Most other studies comparing diagnostic assays

concur with the supreme specificity and sensitivity of the nPCR technique

developed by Chase and Pascho (1998). However, despite its high

specificity and sensitivity, one cannot determine infection intensity based

exclusively on nPCR results. The isolation of *R. salmoninarum* from infected

tissues, in conjunction with the confirmation of representative colonies via

nPCR has also been effective in identifying 76.4% of the infected fish in this

study, although culturing is lower in sensitivity than nPCR alone. Retrieving

R. salmoninarum from tissues by culture alone indicated a presence of at

least 40-100 live bacterial cells/g, but did provide an estimate for the intensity

of infection (Lee 1989; Miriam et al. 1997).

Q-ELISA yielded 67.6% *R. salmoninarum* prevalence, which was lower

than either nPCR (94.2%) or the culture assays (77.2%). Previous studies

estimated that relatively high numbers of bacterial cells (1.3×10^4 bacteria/ml

ovarian fluid, Pascho et al. 1998; and 10^3 cells/g kidney tissue, Jansson et al.

1996) are necessary for the detection of *R. salmoninarum* excreted proteins

via the Q-ELISA assay. This lower sensitivity of Q-ELISA may not be due to

the assay or reagents themselves, but rather to the metabolic activities of *R. salmoninarum* at the time of testing, which influences the amount of bacterial antigens secreted. It is known that *R. salmoninarum* can live within fish tissues for a relatively long period in low numbers and in a quiescent state (Bruno 1986), and that *R. salmoninarum* activation and the secretion of extracellular proteins do not take place in infected fish all of the time. The inherent advantage of Q-ELISA is that this technique allowed us to recognize that the majority Q-ELISA positive fish had relatively low concentrations of *R. salmoninarum* antigen (83.5%, table 1). Only a minority of fish exhibited medium (6.5%) or high (10%) antigen concentrations. Low *R. salmoninarum* antigen concentrations indicated the presence of relatively lower numbers of *R. salmoninarum* in a less active metabolic status when compared to fish with higher *R. salmoninarum* antigen concentration, a matter that could be associated with either early or late stages of infection (Sami et al. 1992; Jónsdóttir et al. 1998).

The lack of agreement in results of the three assays is difficult to explain, however, not surprising since the fish were naturally, rather than experimentally infected. Naturally infected fish of this study were likely at different phases of *R. salmoninarum* infection at the time samples were collected. This factor may have contributed to the appearance of diverse diagnostic patterns that ranged from full agreement among the three diagnostic assays (e.g., Patterns 3 and 6) to a more unexpected pattern (e.g., Pattern 5). Other factors that likely have contributed to the diversity of

diagnostic patterns include differences in the *R. salmoninarum* dose to which

the fish were exposed, pathogenicity of specific *R. salmoninarum* strains, fish

immunologic status, and individual genetic susceptibility.

Careful examination of the six patterns reveals what appears to be a

logical progression of infection, with each of the patterns representing a

probable stage along the course of *R. salmoninarum* infection. Pattern 1 is

most likely the initial stage of infection establishment within the kidney, with a

minimal number of bacteria localized in tissues. Pattern 2 indicates that the

infection has been established and bacterial numbers are high enough to be

isolated on the MKDM medium. Pattern 3 is the most common, with *R.*

salmoninarum antigens exceeding the detection limit of Q-ELISA. In the fish

in which the infection has progressed, *R. salmoninarum* antigen

concentrations increase from low to medium to high. While high *R.*

salmoninarum antigen concentrations are a strong indicator of active, well-

established infections that may lead to clinical cases with mortalities, it does

not necessarily indicate the presence of the characteristic clinical and

pathological manifestations of BKD, including granuloma formation (Miriam et

al. 1997; Jónsdóttir et al. 1998). This is most likely due to the fact that *R.*

salmoninarum soluble antigens suppress a number of fish immune defense

mechanisms and thereby host reactions to infection may be lacking (Turaga

et al. 1987; Wiens and Kaattari, 1991; Fredricksen et al. 1997, Densmore et

al., 1998, Jónsdóttir et al., 1998; Grayson et al. 2002).

Pattern 4 may represents fish that appear to be recovering from *R. salmoninarum* infection, as viable bacteria present in their tissues were not plentiful enough to be isolated, yet bacterial DNA and *R. salmoninarum* antigens continue to be present. Fish in Pattern 5 are possibly in an advanced stage of recovery, with only minute traces of *R. salmoninarum* antigens remaining. Indeed, all 14 fish in this pattern exhibited Q-ELISA values that neared those of the negative control. *Renibacterium salmoninarum* antigens form immune complexes that deposit in the kidney glomeruli and are eliminated slowly through the kidneys (Sami et al. 1992). Fish in Pattern 6 were either never exposed to *R. salmoninarum*, refractory to infection, or were infected and then totally eliminated *R. salmoninarum* and its antigen from their systems. Since *R. salmoninarum* is widespread in Michigan waters, it is more likely that fish in this group have been exposed to *R. salmoninarum* before testing. Further restoration and conservation efforts should focus on increasing the proportion of this pattern in salmonid populations.

Findings suggest that fish stocks tested in this study are not uniform in the distribution of patterns (Figures 6-9). For example, most of the tested LMRW chinook salmon and PRW MI-adapted coho salmon (both returning from Lake Michigan) were either in pattern 2 (>25%) or 3 (>50%), albeit with low antigen concentrations. It is likely that *R. salmoninarum* infection in these two Lake Michigan stocks seldom progress. Patterns of SRW chinook salmon and captive lake trout were evenly distributed, indicating an ongoing

infection with many fish recovering. In the case of Hinchenbrook coho salmon and captive brook trout, both prevalence and intensity were high, with very few fish in patterns 4-6. Indeed, in both these stocks, overt clinical signs of BKD and mortalities (in the case of brook trout) are often observed (unpublished observations). Brook trout are known for their high susceptibility to *R. salmoninarum* infection (Snieszko and Griffin 1955; Mitchum et al. 1979), Hinchenbrook coho salmon strain were introduced to the Great Lakes basin from New York State relatively recently (G. Whelan, Michigan Department of Natural Resources, personal communication) and have proven to be more susceptible to *R. salmoninarum* infection when compared to the Michigan-adapted coho salmon strain that was introduced to the Great Lakes basin in 1967 (Borgeson 1970; Keller et al. 1990).

While the explanations provided herein may logically illustrate the course of *R. salmoninarum* natural infection, it should be emphasized that the data of this study were generated using kidney tissues only. Kidneys are the primary targets of *R. salmoninarum* (Fryer and Sanders, 1981); however, other organs should also be assessed in future studies to better understand BKD pathogenesis, particularly in natural infections. So far, the relatively few studies addressing BKD course and progression of infection relied upon experimental infection (Flaño et al 1996, White et al. 1995). Moreover, further correlation of diagnostic testing patterns with clinical observations and tissue alterations in stained sections are needed to better evaluate impacts of *R. salmoninarum* infection at the population level.

Fish & source**	# fish tested	Frequencies of different diagnostic testing patterns*					
		Pattern 1	Pattern 2	Pattern 3	Pattern 4	Pattern 5	Pattern 6
LM-CH	42	0 (0 %)	11/42 (26 %)	25/42 (59.5 %)	1/42 (2.4 %)	5/42 (12 %)	0 (0 %)
SR-CH	58	15/58 (26 %)	15/58 (26%)	12/58 (20.7 %)	8/58 (13.8 %)	5/58 (8.6 %)	2/58 (3.4 %)
PR-CO	131	3/131 (2.3 %)	34/131 (26%)	75/131 (57.2 %)	18/131 (13.7%)	0 (0 %)	2/131 (1.5 %)
PR-HB	53	1/53 (1.9 %)	8/53 (15%)	43/53 (81.1 %)	1/53 (1.9 %)	0 (0 %)	0 (0 %)
MBKT	41	4/41 (9.8 %)	2/41 (4.9 %)	26/41 (63.4 %)	8/41 (19.5 %)	1/41 (2.4 %)	0 (0 %)
MLT	39	1/39 (2.6 %)	17/39 (43.5 %)	13/39 (30.8 %)	2/39 (5 %)	3/39 (7.7 %)	3/39 (7.7 %)
Total	364	24 (6.7 %)	87 (23.9 %)	194 (53.3 %)	38 (10.4 %)	14 (3.8 %)	7 (1.9 %)

Table 9. Frequencies of different diagnostic testing patterns of salmonid feral spawners and captive broodstocks collected from different geographical locations in Michigan during fall 2002.

* Diagnostic testing patterns: Refer to different possible combinations of nPCR, Q-ELISA and Culture results:

Pattern 1:   PCR (+) Q-ELISA (-) Culture (-)
Pattern 2:   PCR (+) Q-ELISA (-) Culture (+)
Pattern 3:   PCR (+) Q-ELISA (+) Culture (+)
Pattern 4:   PCR (+) Q-ELISA (+) Culture (-)
Pattern 5:   PCR (-) Q-ELISA (+) Culture (-)
Pattern 6:   PCR (-) Q-ELISA (-) Culture (-)

** Fish & source:

LM-CH: Chinook salmon collected from Little Manistee Weir
SR-CH: Chinook salmon collected from Swan River Weir
PR-CO: Michigan adapted coho salmon collected from Platt River Weir
PR-HB: Hinchenbrook coho salmon collected from Platt River Weir
MBKT: Brook trout collected from Marquette State Fish Hatchery
MLT: Lake trout collected from Marquette State Fish Hatchery
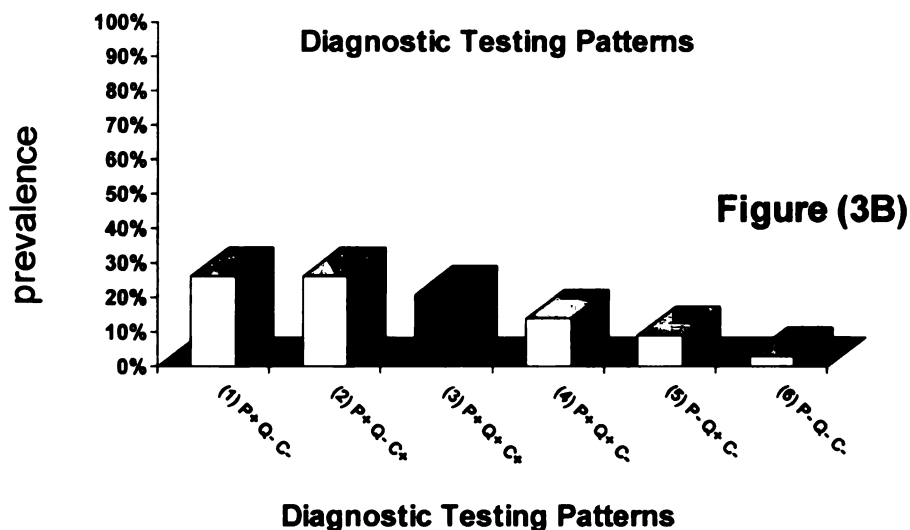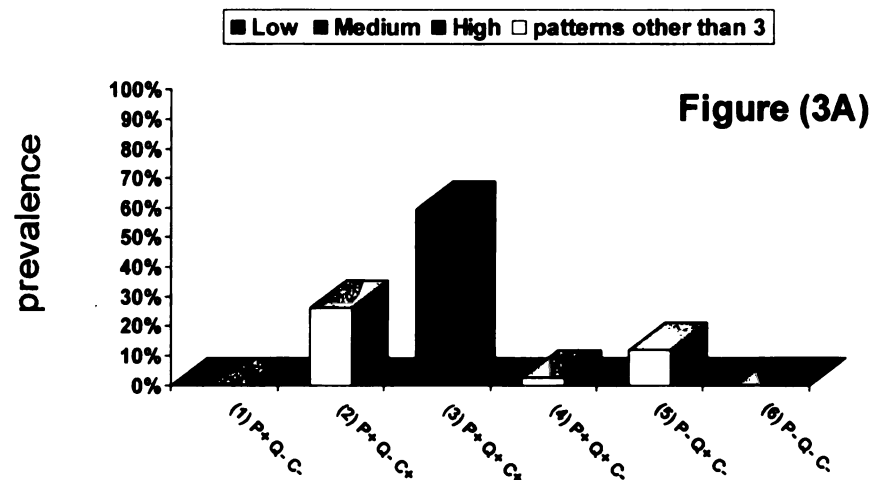
Figure 3. Frequencies of different diagnostic testing patterns of feral chinook salmon spawners.

Note: 3A: Chinook salmon from Little Manistee Weir
    3B: Chinook salmon from Swan River Weir
    Diagnostic testing patterns: P (nPCR), Q (Q-ELISA) and C (Culture).
    Clear columns represent any diagnostic testing pattern other than pattern (3)
    Colored column represents pattern (3) with three colors referring to different Q-ELISA intensities in this pattern. Intensity was considered high if the antigen concentration ≥ 1; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.

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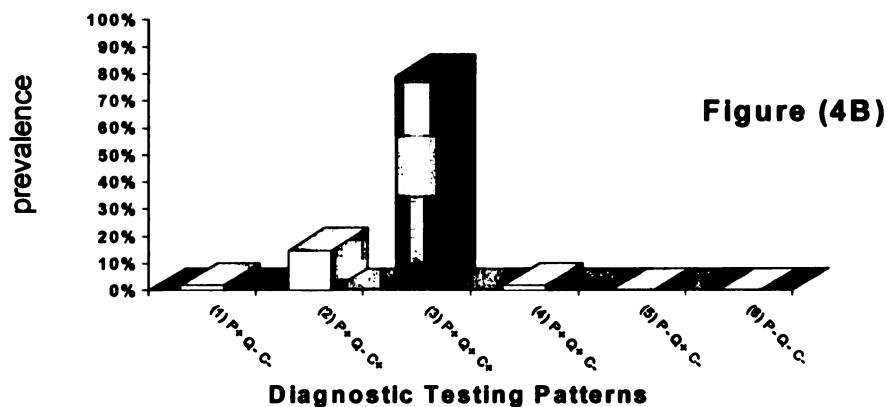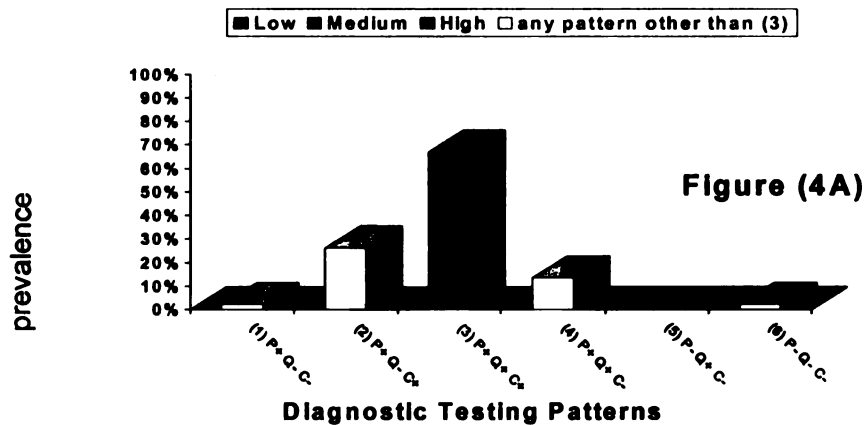
Figure 4. Frequencies of different diagnostic testing patterns of feral coho salmon spawners collected from the Platte River Weir

Note: 4A: Michigan adapted coho salmon
4B: Hinchenbrook coho salmon
Diagnostic testing patterns: P (nPCR), Q (Q-ELISA) and C (Culture)
Clear columns: represent any diagnostic testing pattern other than pattern (3)
Colored column: represents pattern (3) with three colors referring to different Q-ELISA intensities in this pattern. Intensity was considered high if the antigen concentration ≥ 1; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.

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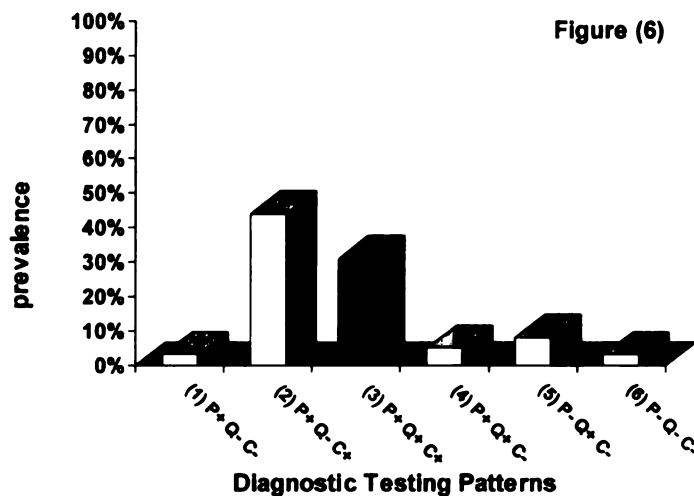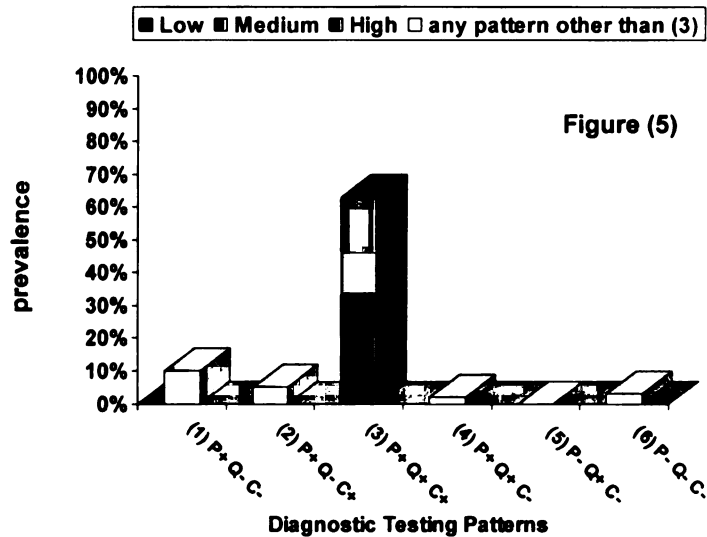
Figure 5 - 6. Frequencies of different diagnostic testing patterns of captive hatchery broodstock.

Note: Figure 5: Brook trout
Figure 6: Lake trout
Diagnostic testing patterns: P (nPCR), Q (Q-ELISA) and C (Culture)
Clear columns: represent any diagnostic testing pattern other than pattern (3)
Colored column: represents pattern (3) with three colors referring to different Q-ELISA intensities in this pattern. Intensity was considered high if the antigen concentration ≥ 1; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.

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CHAPTER FOUR

CURRENT STATUS OF *RENIBACTERIUM SALMONINARUM* INFECTION IN CHINOOK (*ONCORHYNCHUS TSHAWYTSCHA*) AND COHO (*ONCORHYNCHUS KISUTCH*) SALMON AT MICHIGAN WEIRS AND HATCHERIES

ABSTRACT

Since the recent introduction of chinook and coho salmon to Lake Michigan in the late 1960s, number of Bacterial Kidney Disease (BKD) epizootics has resulted in mass dieoffs among the feral spawner stage of these fish. The first epizootic occurred among the coho salmon spawners in 1967 while the latest occurred among the same stage of chinook salmon throughout the period between late 1980s and early 1990s. Management of the disease by hatchery personnel and natural resources managers attempting to interrupt the cycle of infection and focus on two life stages; namely, the gamete-collection stage and the fry/fingerling stage while reared at state fish hatcheries prior to their release.

Data generated in this study provide additional evidence that (*Renibacterium salmoninarum*) *R. salmoninarum* infection is enzootic in Michigan and widespread in feral stocks of chinook and coho salmon in both lakes Michigan and Huron. Lake Michigan's salmon tend to have an overall higher prevalence than that observed in Lake Huron's fish. *Renibacterium salmoninarum*

prevalence in returning feral chinook salmon seem to fluctuate among years, although a definitive decrease can be observed since the start of this study in 2001. However, both strains of coho salmon spawners showed a steady decline in *R. salmoninarum* prevalence throughout the 4 years period. Data of this study demonstrates the ability of both chinook and coho salmon females and males to shed *R. salmoninarum* through the ovarian fluid and milt. Results also indicated that over 90% of female and 80% of male chinook salmon tested in this study from both watersheds were not shedding *R. salmoninarum* antigens along with their gametes. The case was different in coho salmon as both male and females shed *R. salmoninarum* antigens at different rates. The *R. salmoninarum* antigen concentrations in Hinchenbrook gametes were significantly higher than in the MI-adapted gametes.

The BKD testing of both feral spawners and their hatchery raised offspring fingerlings demonstrates that the current testing and culling programs have been successful in reducing *R. salmoninarum* transmission. This study also demonstrates that some hatchery practices were more effective than others in controlling the spread of *R. salmoninarum* and that a reliable improvement in the reduction of *R. salmoninarum* prevalence within the hatcheries involved in this study has been achieved.

INTRODUCTION

Over the last 150 years, several attempts have been made to introduce Pacific salmon species into the Great Lakes. The Michigan Department of Natural Resources (MDNR) initiated the most recent and successful of these introductions in 1966 for coho salmon (*Oncorhynchus tshawytscha*) and 1967 for chinook salmon (*Oncorhynchus* kisutch). In 1966, MDNR stocked a total of 659,000 coho salmon yearlings in a tributary of the Manistee River and the Platte River (Borgeson 1970). In 1967, similar number of chinook salmon smolts from the west coast was introduced into Lake Michigan (Keller et al.1990). After introduction, both chinook and coho salmon spread throughout the Great Lakes basin where they have become the most popular sport and commercial fishery in the State of Michigan (Dexter and O'Neal 2004).

Currently, the Maintenance of the salmon population in the Great Lakes is primarily dependent upon the stocking of hatchery-propagated fish. This practice requires the collection of gametes at the weirs from the fall returning runs, the raising of the offspring at state hatcheries, and the subsequent release of fingerlings at a weight 4-6 g/fish (Dexter and O'Neal 2004). The stocked fingerlings move to the smolt stage at the various stocking sites in the rivers, and then begin their migration to the Great Lakes (Seelbach 1985). Three to four years later, spawning runs of salmon return with high fidelity to the Michigan

streams in which they were either stocked or spawned. Once either natural or man-assisted spawning has commenced, the fish perish (Dexter et al. 2004).

In 1967, multiple cases of coho salmon mortalities were reported by anglers along the shores of Lake Michigan. MacLean and Yoder (1967) confirmed the presence of clinical BKD in adult coho salmon that were dying along the shores of Lake Michigan. The authors also reported that Lake Superior coho salmon suffered higher BKD prevalence than those of Lake Michigan and the males had higher prevalence than females. In 1986, clinical cases of were also observed in spawning chinook salmon runs at the Little Manistee River Weir, Michigan. The disease became more prevalent in subsequent years, and incidence ranged from 53-100% during 1986-1991 (Holey et al. 1998, Hnath and Faisal 2005). Concomitant with the BKD surge occurring at the weirs, chinook salmon die-offs were reported in 1986 and 1987 and reached a peak in 1989. Minimum estimates of 20,000 adult fish were found dead (Johnson and Hnath 1991). The majority of dead fish exhibited lesions indicative of BKD, hence BKD was considered to be a major contributing factor to the chinook salmon kills. Starliper et al. (1997) demonstrated that the *R. salmoninarum* isolates associated with chinook and coho salmon mortalities in Michigan are more virulent than those retrieved from other regions in North America.

Renibacterium salmoninarum is an obligate intracellular pathogen that is transmitted both horizontally (Mitchum and Sherman 1981; Bell et al 1984) and vertically (Evelyn et al. 1984, 1986). *Renibacterium salmoninarum* pathogenicity relies upon a number of extracellular proteins (ECP) that possess

immunosuppressive, proteolytic, hemolytic, and DNA degrading activities (Austin and Rodgers 1980; Bruno and Munro 1986b; Turaga et al. 1987; Fredriksen et al. 1997). When ECP alone were injected into susceptible fish, 80-100% mortalities may occur (Shieh 1988). The ECP contain a water-soluble, cell surface, 57- kDa protein ($p57$) that has been demonstrated to be a major virulence factor of *R. salmoninarum* (Getchell et al. 1985; reviewed in Wiens and Kaattari 1999).

The occurrence of vertical transmission of *R. salmoninarum* is well documented (Evelyn et al. 1984; Lee and Evelyn 1989), but believed to be rare (Hamel 2001). It seems that inclusion of *R. salmoninarum*-ECP into the eggs increases the susceptibility of the offspring to *R. salmoninarum* infection (Brown et al.1996). The studies of Brown et al. (1996) provided evidence that *intra-ovum* inclusion of p57 can result in decreased immune functions when the resulting fry are exposed to *R. salmoninarum*. The likelihood of vertical transmission of the disease (from parent to offspring) or *intra-ovum* antigen inclusion immunotolerance is related to the ovarian fluid infection levels (Hamel 2001). Another complication results from the fact that the entry of *R. salmoninarum*-ECP may cause life-long immunotolerance of the disease, greatly decreasing the probability of survival to spawning (Hamel 2001).

Because *R. salmoninarum* can be transmitted vertically, MDNR adopted a stringent visual inspection on each spawning female and the subsequent culling of any gametes previously collected from spawning females showing overt signs of clinical BKD. In addition, kidney swabs of individual fish were routinely examined for the presence of *R. salmoninarum* soluble antigens using a

monoclonal antibody based Field ELISA (FELISA) (Gary Whelan, MDNR

personal communication). This rapid field test allowed the detection of fish whose

kidneys are laden with *R. salmoninarum* antigen, and exclusion of their eggs

from further incubation (Beyerle and Hnath 2002; Hnath and Faisal 2005).

Neither health inspection, nor FELISA were performed on males because the

published studies of Klontz et al. (1983) and Evelyn et al. (1986) minimized the

role that male fish play in the vertical transmission of *R. salmoninarum*.

In the Great Lakes, chinook and coho salmon are not in their native range,

yet many of the stocked fish are able to survive and return to rivers to

successfully spawn. How returning salmon were able to co-exist with *R.*

salmoninarum in a non-native range is currently unknown and deserves further

investigation. This is particularly important since most of our knowledge on *R.*

salmoninarum-returning spawner salmon interactions originated from studies

performed in the Pacific Northwest, where the fish alternate between marine and

freshwater environments. Hence, the major thrust of this chapter is to identify

some of the basics of the *R. salmoninarum-* returning spawner salmon

relationship in Michigan waters such as the prevalence and intensity of *R.*

salmoninarum infection in returning spawning runs, the contribution of both males

and females to shedding of *R. salmoninarum* and its immunosuppressive soluble

antigens. Additional aim of this study is to determine the efficacy of the culling

program and hatchery practices on minimizing vertical and horizontal

transmission of *R. salmoninarum*.

MATERIALS AND METHODS

Fish and sampling. In this study, samples were collected from three major egg-take weirs, and three state fish hatcheries in Michigan (table 10). Chinook salmon samples from returning spawners were collected from the Little Manistee River Weir (LMRW, Manistee County, Lake Michigan watershed) and Swan River Weir (SRW, at Rogers City, Presque Isle County, Lake Huron watershed). LMRW is the major chinook salmon egg collection operating weir on the Michigan-side of Lake Michigan, situated on the Little Manistee River, a major trout tributary that annually receives millions of fingerlings via stocking. Swan River Weir is the only egg-take facility on Lake Huron and is considered an ideal tributary for the further development of pre-smolts to the smolt stage before emigration to Lake Huron (Dexter and O'Neal, 2004).

Coho salmon samples were collected from two strains of coho salmon; the Michigan-adapted and the Hinchenbrook strains. Adult coho salmon spawning runs were sampled at the Platte River Weir (PRW), off the Platte Bay, at Beulah, MI (Lake Michigan watershed) during egg-takes in the falls of 2001 through 2004. Platte River Weir is the only operating egg-take weir on the Michigan-side of Lake Michigan that is used exclusively for collecting coho salmon gametes.

For the purpose of this study, kidney tissues were sampled from 303 and 653 chinook salmon spawners at the LMRW and SRW respectively (table 10). A total of 383 and 273 kidney samples were sampled from Michigan adapted coho and Hinchenbrook coho salmon spawners respectively at the PRW (table 10).

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Additionally, in 2004, ovarian fluid was collected from 280 and 60 chinook salmon females at SRW and LMRW respectively. Moreover, ovarian fluid was also collected from 60 Michigan adapted and 60 Hinchenbrook coho females at PRW. Concomitantly, milt from 60 LMRW chinook, 60 PRW Michigan adapted coho, and 60 PRW Hinchenbrook coho male spawners were collected in 2004.

Following an approximate 8 (for chinook salmon) and 18 (for coho salmon) month period of egg incubation and fish rearing in three state fish hatcheries (Wolf Lake state hatchery (WLH) in Mattawan city, Platte River state hatchery (PRH) in Beulah city, and Thompson state hatchery (THH) in Manistique, Michigan), kidney tissues of juvenile fingerlings were collected prior to their release into the basins of lakes Michigan and Huron and subsequently analyzed for the presence of *R. salmoninarum*. Within the period of 2002-2005, a total of 965 chinook and 480 coho salmon kidney samples were collected from juvenile fingerlings at WLH (360 chinook salmon), PRH (425 chinook and 480 coho salmon), and THH (180 chinook salmon) (table 11).

The sacrifice of feral spawners entailed exposing the fish to carbon dioxide-laden water, followed by a blow to the head. Following gamete collection, the abdominal cavity was cut open to examine individual internal organs for signs associated with BKD, followed by the collection of approximately one gram of tissue from anterior, posterior and middle kidney sections. Cross contamination was avoided by using sterile dissecting tools for each fish. Coelomic fluid samples were collected using sterile transfer pipettes from the egg/ovarian fluid mixture, transferred to 5 ml sterile polypropylene tubes, and kept on ice until

processed at the laboratory (Michigan State University, East Lansing, MI). Milt samples were collected directly from the middle stream of milt into sterile 10-ml polypropylene tubes. Fingerlings were euthenized by immersion in an overdose of anesthesia using MS-222 (tricaine methan sulfonate, Finquel- Argent Chemical Laboratories, Washington).

Sampling and sample processing: Samples from fish were analyzed individually unless otherwise indicated. Kidney samples from the anterior, posterior, and middle sections of the kidney were transferred in sterile 7.5 cm x 18.5 cm Whirl Pak® bags (Nasco, Forte Atkinson, WI), kept on ice, and were softened as much as possible through multiple cycles of physical pressure. The homogenized kidney tissues were diluted 1:4 (w/v) with Hank's Balanced Salt Solution (HBSS, Sigma Chemical Co, St. Louis, MO, USA) and then stomached for 2 minutes at high-speed using the Biomaster Stomacher-80 (Wolf Laboratories Limited, Pocklington, York, UK). In the case of either milt or ovarian fluid, 1 ml of each sample was diluted 1:2 (v/v) in HBSS for Q-ELISA.

Measurements of *Renibacterium salmoninarum* antigen using the Quantitative Enzyme-linked Immunosorbent Assay (Q-ELISA). Aliquots of 250 µl of each sample were transferred into 1.5 ml safe lock microfuge tubes, to which an equal volume of 0.01 M Phosphate Buffered Saline Tween 20 (0.05 % PBS-T20) (Sigma) with 5 % natural goat serum (Sigma) (Olea et al. 1993) and 50 µl CitriSolv solution (Fisher Chemicals, Fairlawn, New Jersey, USA)

(Gudmunsdottir et al. 1993) were added. The solution was then thoroughly vortexed, incubated at 100 °C on heat blocks with a rotary shaker for 15 minutes, followed by 2 hours of incubation at 4 °C. After incubation, the mixture was centrifuged at 6000g for 15 minutes at 4 °C. The aqueous supernatant of each sample was carefully collected and then transferred to a 1.5 ml microfuge tube for Q-ELISA. The Q-ELISA method used in this study was described by Pascho and Mulcahy (1987); Alcorn and Pascho (2000). The positive-negative threshold absorbances were calculated according to the method described by Meyers et al. (1993) .The positive–negative cutoff absorbance for the kidney homogenate was 0.10. The Q-ELISA positive samples were assigned the following antigen level categories: low (0.10 to 0.19), medium (0.20-0.99) and high (1.000 or more) as developed by Pascho et al. (1998). Intensity of infection among certain group of fish is determined by the prevalence of samples that possess high Q-ELISA antigen concentrations.

Statistical Analysis:

Due to the nature of this study, descriptive statistics were heavily relied upon. For year-to-year, salmon strains differences, gamete source, and hatchery comparisons, the data was tested for normality, and then analyzed by an analysis of variance (ANOVA), student t test (parametric) or Mann-Whitney Rank Sum (nonparametric) Tests with an alpha = 0.05 (P=0.05).

RESULTS

1. *Renibacterium salmoninarum* prevalence and intensity in kidney tissues of returning salmon spawners. *Renibacterium salmoninarum* antigens have been detected in the kidneys of chinook and coho salmon returning to both weirs in every year sampling took place. Generally, there was a slight decline in prevalence and intensity when the data of 2004 was compared with that of 2001.

a. Chinook salmon returning spawners. The prevalence of *R. salmoninarum* in chinook salmon returning to the Little Manistee Weir (LMRW) gradually decreased from 84% in 2001 to 20 % in 2003, before abruptly rising to 67% in 2004. Additionally, *R. salmoninarum* intensity from the same fish groups declined from 5% in 2001 to 0% in 2002, and then increased slightly to 2.5% in 2003 and 2004. The prevalence of *R. salmoninarum* in the Swan River Weir (SRW) constantly declined, but the intensity followed a different trend. The prevalence of *R. salmoninarum* in the SRW chinook salmon declined from 46% in 2002 to 12.3 % in 2004. However, *R. salmoninarum* intensity in the SRW fish showed a slight decrease from 5% in 2002 to 3% in 2003, with a subsequent increase to 4% in 2004 (Figure 7). Comparison of the prevalence and intensity of *R. salmoninarum* in the feral spawner chinook from the two weirs demonstrated a consistently higher prevalence in the LMRW chinook salmon, with the exception of the samples taken in 2003. On the contrary, the intensity of the disease seemed to be higher in the SRW salmon over the same period of analysis (Figure 7). However, statistical analysis of the data revealed that difference

between the prevalence of *R. salmoninarum* in chinook salmon collected from the two different weirs and at the same hatchery in different years of collection was insignificant.

To determine sex-related differences among chinook salmon from LMRW and SRW, *R. salmoninarum* prevalence and intensity was determined in separate groups of males and females returning to both weirs in 2002 and 2004. Data showed no statistically significant differences between males and females from both weirs, although there was a trend for males to have higher prevalences and intensities of infection as compared to females (Figure 10).

b. Coho salmon returning spawners. The prevalence of *R. salmoninarum* in Michigan-adapted coho strain decreased from 84% in 2001 to 64.8 % in 2002 then to 40 % in 2003 ending with 24.2 % in 2004. Additionally, *R. salmoninarum* intensity from the same fish group showed no marked difference through the period between 2001 and 2004 (Figure 8). The prevalence of *R. salmoninarum* in the Hinchenbrook strain has decreased from 100 % in 2001 to 82 % in 2002 then to 29 % in 2003, before rising to 72.5% in 2004. The intensity of infection in the same group of fish followed a similar trend in which the intensity has decreased from 49 % in 2001 to 21.4 % in 2002 then to 10.4 % before sharply increasing to 47.5 % in 2004 (Figure 8). Comparison of the prevalence and intensity of *R. salmoninarum* in spawners of both coho salmon strains demonstrates a consistently higher prevalence and intensity in the Hinchenbrook coho with the exception of the samples taken in 2003. Additionally, clinical examination of the Hinchenbrook, but not the MI-adapted, strain revealed

the presence of signs characteristic of BKD. Statistical analysis showed statistical difference (P< 0.05) increases in both prevalence and intensity of *R. salmoninarum* over the years in the Hinchenbrook strain as compared to the MI-adapted strain. Moreover, data showed no statistically significant differences between males and females from both weirs

2. *Renibacterium salmoninarum* prevalence and Intensity in ovarian fluid and milt of returning salmon spawners. The shedding of *R. salmoninarum* with the gametes was tested in 2004 returning spawners. As displayed in tables 12, 13 and 14, *R. salmoninarum* and its soluble antigens were passed along with the gametes of both sexes albeit not in all of the infected fish.

a. Chinook salmon returning spawners. While 28/60 (47%) LMRW female salmon were infected; only 6 of these fish had *R. salmoninarum* antigens in their ovarian fluids (table 13). Similarly, only half of the infected SRW females exhibited *R. salmoninarum* antigens in their ovarian fluids. The same trend was noticed in the LMRW males, with only 20% of infected fish had *R. salmoninarum* antigens in their milt.

Table 13 summarizes the difference in prevalence between kidneys and gametes of the same fish. More than 80% of the fish produced *R. salmoninarum*-free gametes (sum of groups 1 and 2 in table 13), even though some of these fish had *R. salmoninarum* in their kidneys. Indeed, a few of these fish exhibited medium and high *R. salmoninarum* antigen titers in the kidneys. In the case of LMRW males, the concentrations of *R. salmoninarum* soluble

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antigens in milt as determined by Q-ELISA coincided with that found in the kidney tissues in all individual fish. Similar findings were observed in the LMRW, with the exception of females# 5 and 29, which had very high *R. salmoninarum* antigen concentrations in the ovarian fluid, while their kidney had low antigen concentrations. Another LMRW female (# 43) shed the *R. salmoninarum* antigen in a very low level in the ovarian fluid, while the kidney tissues tested negative. A similar trend was noticed with the SRW chinook salmon, where 18 females shed the *R. salmoninarum* antigen in the ovarian fluid, while the majority of females exhibited a comparable titer of *R. salmoninarum* antigens in both ovarian fluids and kidneys. Five fish (1.8%) shed the antigens in the ovarian fluids while their *R. salmoninarum* antigen in their kidney was below the detection level (Group 4 in table 13).

b. Coho salmon returning spawners. Although 24.2% of the MI adapted coho strain was infected, only 11.7 % of these fish had *R. salmoninarum* antigens in their ovarian fluids. Similarly, while 72 % of the MI adapted coho strain was infected, only 50 % of these fish had *R. salmoninarum* antigens in their ovarian fluids (table 12). The same trend was noticed in the MI adapted coho males, with only 5% of infected fish having *R. salmoninarum* antigens in their milt samples. However, results demonstrated that the Hinchenbrook coho males shed more *R. salmoninarum* and its antigens (55 %) in milt than that of the MI adapted strains (5 %) (tables 12 and14). The same trend was noticed when comparing the prevalence and intensity of *R. salmoninarum* antigens shed along with ovarian fluid of females of both strains (tables 12 and 14).

3. *Renibacterium salmoninarum* prevalence and intensity in kidney tissues of hatchery-raised juvenile fingerlings. The prevalence of BKD in juvenile spring salmon (originally hatched from the fall returning spawner parents) raised in three hatcheries was followed for four consecutive years (2002-2005; tables 15-16 and Figure 11). In general, *R. salmoninarum* prevalence and intensity infection were lower in offspring when compared to parent stocks.

a. Chinook salmon juvenile fingerlings. In case of chinook salmon, this relationship was not present when the results of *R. salmoninarum* testing were grouped by individual hatchery. However, offspring raised in certain hatcheries tended to exhibit a consistent pattern. For example, chinook salmon fingerlings raised in Thompson state fish hatchery (THSFH) exhibited the lowest prevalence and intensity of *R. salmoninarum* in kidneys when compared to their cohorts raised in other hatcheries. Year to year comparison, however, showed that over the years, *R. salmoninarum* prevalence has declined in the other two hatcheries as well. For example, *R. salmoninarum* prevalence in spring chinook salmon fingerlings, originally spawned at LMRW and then raised in WLSFH, declined from 100% in 2002 to 5% in 2005 (table 15). Similarly, the prevalence of spring chinook salmon originally spawned at LMRW and raised at PRSFH declined from 100% in 2003 to 0% in 2004 and 5% in 2005. Likewise, spring chinook salmon originally spawned from SRW parents and hatched in WLSFH showed a decline in BKD prevalence from 35% in 2004 to 10% in 2005 (table 16). Sharp decreases

in BKD prevalence was also noticed in the spring Chinook salmon from PRSFH between 2003 and 2004 (92% vs. 0%) before slightly increasing to 8.3% in 2005. However, statistical analysis revealed non-significant differences in prevalence and intensity of infection when year-to-year comparison or hatchery-to-hatchery comparisons were adopted.

b. Coho salmon juvenile fingerlings. The prevalence of BKD in spring coho salmon juvenile fingerlings raised in the Platte River State Fish Hatchery was followed up for three consecutive years (2003-2005). In general, *R. salmoninarum* prevalence and intensity was lower in offspring when compared to parent stocks (Figure 11). Year to year comparison, however, showed that over the years, *R. salmoninarum* prevalence has steadily declined in the two coho salmon strains. For example, *R. salmoninarum* prevalence in MI adapted coho salmon fingerlings, originally spawned from MI adapted coho salmon spawners at PRW and then raised in PRSFH, declined from 50 % in 2003 to 27 % in 2004 ending with 6.7 % in 2005. The same trend was noticed among the Hinchenbrook coho fingerlings raised at the same hatchery in which the prevalence was steadily decreased from 75 % in 2003 to 22 % in 2004 ending with slight decline to 20 % in 2005. Comparing the *R. salmoninarum* prevalence in the two strains of hatchery-raised coho salmon to that of their corresponding parents demonstrated that fingerlings follow a statistically significant (P<0.05) decline in the 2004 and 2005 samples of both strains. Data also demonstrated that Hinchenbrook coho salmon fingerlings possessed statistically significant

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higher *R. salmoninarum* prevalence (P<0.05) than the MI-adapted coho strain

through the 3 years testing period.

DISCUSSION

Data generated in this study provide additional evidence that *R. salmoninarum* infection is enzootic in Michigan and widespread in feral stocks of feral spawner salmon in both lakes Michigan and Huron. As displayed in Figure 7, Lake Michigan's Chinook salmon tends to have an overall higher prevalence than that observed in Lake Huron's Chinook salmon. In the case of SRW chinook salmon, prevalence was much lower and intensity was relatively higher when compared to the LMRW salmon. The stocking of chinook salmon in Lake Huron is a relatively new event that started in the 1990s, unlike in Lake Michigan, where chinook salmon have been stocked since 1967 (Dexter and O'Neal, 2004). The recent Chinook salmon introduction in Lake Huron has most likely contributed to the relatively low *R. salmoninarum* infection prevalence observed in this study. *Renibacterium salmoninarum* infection prevalence and intensity in returning feral chinook salmon seem to fluctuate among years, although a reliable decrease can be observed since the start of this study in 2001. However, the unexpected surge in prevalence recorded in the 2004-LMRW salmon is alarming. A similar increase in *R. salmoninarum* prevalence was observed in the Lake Michigan stock of Hinchenbrook coho salmon, a strain of coho salmon that is especially susceptible to *R. salmoninarum*. Further studies are needed in forthcoming years to monitor *R. salmoninarum* infection levels in Lake Michigan salmon stocks.

Although MI-adapted and Hinchenbrook strains belong to the same species and residing in the same watershed, they exhibited marked differences in *R. salmoninarum* prevalence and intensity. There are no published reports on *R. salmoninarum* infection in the Hinchenbrook strain from New York, from which it was imported, yet this strain is known in New York for its marked growth, high survival and return rates (John Shachte, personal Communication). The susceptibility of the Hinchenbrook strain to *R. salmoninarum* may be related to its recent introduction into Lake Michigan where BKD is enzootic. The decrease in *R. salmoninarum* prevalence over the years is a good indication of a beginning of adaptation of the strain to its new environment. When the MI-adapted strain was first introduced from the Pacific Northwest, BKD-related epizootics were reported along the shores of Lake Michigan (MacLean and Yoder, 1967). Adaptation in *R. salmoninarum*-contaminated environments has been reported in the Arctic char and the brown trout in Iceland (Jonsdóttir et al. 1998). Genetic make up of the two strains may have also played a role in the difference in *R. salmoninarum* susceptibility. Winter et al. (1980) reported the presence of genetic basis for differences in susceptibility to *R. salmoninarum* among of steelhead trout strains. Further, Beacham and Evelyn (1992) also reported the presence of differential susceptibility to *R. salmoninarum* in a number of chinook salmon strains. Other important factors that may have contributed to differences between the two strains is their diverse migration patterns in Lake Michigan (Dexter and O'Neal, 2004).

Based only on the data of this study, it is difficult to determine whether such high levels of *R. salmoninarum* are associated with BKD-related mortalities. The eruption of BKD in *R. salmoninarum* infected fish is influenced by a number of factors including changes in habitat and forage fish availability. As reported from other fish species and pathogens, changes in the spatial distribution of fish and increased density of the fish population can affect the frequency of contact between infected and non infected migrating fish which will be translated to rapid spread and progression of infection (Reno, 1998). For example, the 1989 BKD epizootic in chinook salmon was associated with a sharp decline in forage fish availability (Holey et al.1998).

Combining the results of 2002 and 2004 kidney Q-ELISA data (Figure 9), males tend to have an equal or slightly higher *R. salmoninarum* prevalence than females. In the North Pacific USA, where much of Chinook salmon BKD data has been generated, females seem to have a much higher *R. salmoninarum* prevalence and intensity than males (Evelyn et al. 1986). Most of the studies, however, were performed using experimental infection models.

The gradual decrease of prevalence and intensity among both chinook and coho salmon coho salmon spawners could be due to the culling procedures adopted in weirs which could minimized the possibility of vertical transmission through infected females to their offspring. Also, the application of strict hygienic measures in weirs by rinsing the spawner fish in iodophores (Ross and Smith 1972) before collecting eggs or milt, using sterile utensils for egg-milt mixing and storage could have minimized the bacterial load transmitted to eggs and milt in

weirs. Moreover, efficient weir practices such as incubating fertilized eggs in clean disinfected trays and incubators as well as hardening of eggs in 2-ppm erythromycin solution (Amos 1977), could have reduced the prevalence and intensity of BKD in the production fish before their release into the Platte River (Maule et al.1996).

Results indicated that the 2003 Hinchenbrook coho salmon fingerlings showed higher BKD prevalence (75%) than that of MI-adapted coho (50 %), which appears consistent with the high *R. salmoninarum* prevalence results of their 2001 parents. As demonstrated by Evelyn et al. (1986), the likelihood of vertical transmission in heavily infected salmon is higher than in lightly infected gamete donors. Moreover, data showed that female MI-adapted coho salmon feral spawner showed slightly higher *R. salmoninarum* prevalence than males of the same strain in the 2002 and 2004 samples. Female coho salmon are usually stressed with the relatively large size of their egg-laden ovaries that occupy more than 70 % of the fish body during the spawning season.

Interestingly, the data presented in this study demonstrates the ability of feral spawner chinook and coho females to vertically transmit the bacterium through the ovarian fluid (tables 12-13). This fluid surrounds the eggs once they have been released into the body cavity following ovulation (Evelyn et al. 1986).

Most of the chinook spawner females that shed *R. salmoninarum* in their ovarian fluid tested positive in the kidneys (table 13). Only in <2% of the chinook females, *R. salmoninarum* was shed in the ovarian fluid, but not in the kidneys. There are three explanations for the appearance of this phenotype. First,

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kidneys are infected in levels that are below the Q-ELISA detection limit.

Second, the fish were recovered from a prior *R. salmoninarum* infection, which

left remnants of *R. salmoninarum*-ECP that are known to be excreted in a slow

fashion (Sami et al. 1992). Indeed, *R. salmoninarum* antigen levels in these fish

were extremely low. Last, kidney samples may have given false negative results.

Most important, however, was the fact that over 90% of chinook salmon tested in

this study from both watersheds were not shedding *R. salmoninarum* antigens in

the ovarian fluid, which is much less than originally thought. As a result, high

levels of *R. salmoninarum* prevalence and intensity in hatchery fingerlings cannot

be attributed to vertical transmission alone. Moreover, the slow multiplication of

R. salmoninarum would not allow the infection to develop in < 6 month old

juvenile chinook salmon fingerlings to levels such as those observed In the

fingerlings of 2002 and 2003. Therefore, it is likely that horizontal transmission

plays a role in spreading infection among hatchery-propagated fish. There is no

doubt, however, that the exposure of offspring to the immunosuppressive *R.*

salmoninarum-ECP can predispose the fish to infection with *R. salmoninarum* or

other pathogens.

Data also demonstrated that over half of Hinchenbrook females and males

shed *R. salmoninarum* along with the gametes as opposed to 12% in females

and 5% males of the MI-adapted strain. This extreme difference can account for

the higher prevalence and intensity of *R. salmoninarum* in the Hinchenbrook

strain as compared to the MI-adapted strain. On the contrary, a higher percent of

infected adult Hinchenbrook coho salmon did not shed *R. salmoninarum* despite

the high levels of *R. salmoninarum* intensity in their kidneys (Group 2 in table 13).

By combining fish in Groups 1 and 2 of table 14, it became clear that a very high percent of coho salmon shed *R. salmoninarum*-free gametes, a matter that may lead to a reduction of *R. salmoninarum* infection incidence in the future. Group 4 on the contrary, are fish that shed *R. salmoninarum* antigen along with the gametes despite their non-detectable *R. salmoninarum* levels in the kidneys. Group 4 and group 3 are probably the cause of the continuous presence of *R. salmoninarum* across generations. High portions of the Hinchenbrook females shed *R. salmoninarum* in their coelomic fluid were tested positive in the kidneys. Only in <2% of the Hinchenbrook and MI adapted coho females, *R. salmoninarum* shed in the coelomic fluid, but not in the kidneys.

It has also been demonstrated that males shed *R. salmoninarum* in their milt, sometimes in high concentrations, and thereby may contribute to the spread of *R. salmoninarum* into eggs through the micropyle during fertilization. In addition, the potential exposure of the embryo to immunosuppressive *R. salmoninarum*-ECP from the milt cannot be ignored (Hamel 2001). It should be noted that only 10 out of 53 infected LMRW males shed *R. salmoninarum* antigen in their milt, while the remaining males were able to keep their seminal plasma below the Q-ELISA detection limits. There is a handful of papers that addressed the role of males in the transmission of *R. salmoninarum* in chinook and coho salmon. The studies of Evelyn et al. (1986) and Rocky et al. (1991) suggested that the role of males in infecting eggs is minimal. Data of this study, however, contradicts other reports and demonstrates that males are capable of shedding *R. salmoninarum*

more than females of the same stock. Further, we strongly recommend that the testing and culling policy, currently applied to females only, be expanded to include males.

The BKD testing of both feral spawners and their hatchery raised juvenile fingerlings demonstrates that the current testing and culling programs have been partially successful in reducing *R. salmoninarum* transmission. This study also demonstrates that some hatchery practices were more effective than others in controlling the spread of *R. salmoninarum* and that a reliable improvement in the reduction of *R. salmoninarum* prevalence within the three hatcheries involved in this study has been achieved. The aforementioned assumption coincided with that of Maule et al. 1996 who reported variations in *R. salmoninarum* prevalence among hatcheries in fingerlings derived from the same stock.

Salmon species and source	Date collected	Number of fish tested
Chinook salmon spawners from LMRW	October, 2001	60
	October, 2002	63
	October, 2003	60
	October, 2004	120
Chinook salmon spawners from SRW	October, 2002	59
	October, 2003	34
	October, 2004	560
Michigan adapted coho salmon spawners from PRW	October, 2001	38
	October, 2002	165
	October, 2003	60
	October, 2004	120
Hinchenbrook coho salmon spawners from PRW	October, 2001	49
	October, 2002	56
	October, 2003	48
	October, 2004	120

Table 10. Details of samples collected from spawning chinook and coho salmon returning to egg-take weirs throughout the period from 2001-2005.

LMRW: Little Mansitee River Weir
SRW: Swan River Weir
PRW: Platte River Weir

Rearing hatchery	Date Collected	Egg-take Weir	Number of fish tested
Wolf Lake State Fish Hatchery	March 2002	Little Manistee River Weir	60
	March 2003	Little Manistee River Weir	60
	March 2004	Little Manistee River Weir	60
		Swan River Weir	60
	March 2005	Little Manistee River Weir	60
		Swan River Weir	60
Platte River Weir State Fish Hatchery	March 2002	Little Manistee River Weir	65
	March 2003	Little Manistee River Weir	60
		Swan River Weir	60
	March 2004	Little Manistee River Weir	60
		Swan River Weir	60
	March 2005	Little Manistee River Weir	60
		Swan River Weir	60
Thompson State Fish Hatchery	March 2003	Swan River Weir	60
	March 2004	Swan River Weir	60
	March 2005	Swan River Weir	60
Total			965

Table11. Chinook salmon juvenile fingerlings collected from Michigan state fish hatcheries in the period from 2002-2005.

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Fish species and source	# of tested body fluid samples	Renibacterium salmoninarum prevalence	% Q-ELISA positive titers		
			High	Medium	Low
Chinook salmon form LMRW	60 ovarian Fluid	6/60 (10 %)	1/60 (1.5 %)	1/60 (1.5 %)	4/60 (7 %)
	60 Milt	10/60 (63.5 %)	8/60 (13 %)	2/60 (3 %)	0/60 (0%)
Chinook salmon from SRW	280 ovarian Fluid	18/280 (6.4 %)	11/280 (4 %)	2/280 (0.7 %)	5/280 (1.78 %)
Michigan adapted coho salmon from PRW	60 ovarian Fluid	7/60 (11.7 %)	5/60 (8.3 %)	1/60 (1.7 %)	1/60 (1.7 %)
	60 Semen	3/60 (5 %)	0/60 (0 %)	0/60 (0 %)	3/60 (5 %)
Hinchenbrook coho salmon from PRW	60 ovarian Fluid	30/60 (50 %)	15/60 (25 %)	3/60 (5 %)	12/60 (20 %)
	60 Semen	33/60 (55 %)	4/60 (6.7 %)	9/60 (15 %)	20/60 (33.3 %)

Table 12. *Renibacterium salmoninarum* prevalence and intensity in ovarian fluid and milt of the 2004 chinook and coho salmon spawners.

Data in this table was generated using a polyclonal antibody-based quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was determined by % of Q-ELISA positive samples of the total number of samples tested. Intensity was considered high if the antigen concentration $\geq$; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.

SRW: Swan River Weir.
PRW: Platte River Weir.

Fish Group	LMRW		SRW
	Females	Males	Females
1. Kidneys & Gametes negative	28/60 (46.7%)	7/60 (11.7%)	240/280 (85.7%)
2. Kidneys + & Gametes -	26/60 (43.3%)	43/60 (71.7%)	22/280 (7.9%)
3. Kidneys + & Gametes + with same *Renibacterium salmoninarum* antigen level	5/60 (8.3%)	10/60 (16.6%)	13/280 (4.6%)
4. Kidneys - & Gametes + with very low intensity	1/60 (1.6%)	0/60 (0 %)	5/280 (1.8%)

Table 13. *Renibacterium salmoninarum* prevalence in kidney and gametes of the 2004 chinook salmon spawners.

Data in this table was generated using a polyclonal antibody-based quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was determined by % of Q-ELISA positive samples of the total number of samples tested. Intensity was considered high if the antigen concentration $\geq$; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.

Fish Group	Hinchenbrook		Michigan Adapted	
	Females	Male	Females	Male
1. Kidneys & Gametes negative	10/60 (17%)	15/60 (25%)	40/59 (68%)	45/60 (75%)
2. Kidneys + & Gametes -	17/60 (28%)	15/60 (25%)	11/59 (19%)	11/60 (18%)
3. Kidneys + & Gametes + with same Renibacterium salmoninarum antigen level	32/60 (53%)	25/60 (42%)	6/59 (10%)	1/60 (2%)
4. Kidneys - & Gametes + with very low intensity	1/60 (2%)	5/60 (8%)	1/59 (2%)	2/60 (3%)

Table 14. *Renibacterium salmoninarum* **prevalence in Kidney and gametes of the 2004 coho salmon spawners (Shedding agreements possibilities between kidney and gametes).**

Data in this table was generated using a polyclonal antibody-based quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was determined by % of Q-ELISA positive samples of the total number of samples tested. Intensity was considered high if the antigen concentration ≥; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.

Fish strain	Gamete Donors		Fingerlings tested 8 month following egg-take		
	Year	*Renibacterium salmoninarum* prevalence	Year	Hatchery	*Renibacterium salmoninarum* prevalence
Little Manistee Chinook	2001	81.7 % Total 43.3 % high 8.3 % medium 30.0 % low	2002	PR	62.0 % Total 0.0 % high 0.0 % medium 62.0% low
				WL	100.0 % Total 100.0 % high 0.0 % medium 0.0 % low
	2002	63.5 % Total 0.0 % high 6.3 % medium 57.1 % low)	2003	PR	100.0 % Total 17.0 % high 8.0 % medium 75.0 % low)
				WL	92.0 % Total 0.0 % high 50.0 % medium 42.0 % low
	2003	20.0 % Total 1.7 % high 1.7 % medium 16.7 % low	2004	PR	0.0 % Total 0.0 % high 0.0 % medium 0.0 % low)
				WL	35.0 % Total 2.0 % high 6.7 % medium 27.0 % low
	2004	67.0 % Total 2.5 % high 7.5 % medium 57.0 % low	2005	PR	5.0 % Total 0 % high 0 % medium 5 % low)
				WL	10.0 % Total 0.0 % high 0.0 % medium 10.0 % low

Table 15. *Renibacterium salmoninarum* infection prevalence and intensity among the Little Manistee River chinook salmon feral spawners and their corresponding hatchery raised fingerlings throughout the period from 2001 to 2005.

Data in this table was generated using a polyclonal antibody-based quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was determined by % of Q-ELISA positive samples of the total number of samples tested. Intensity was considered high if the antigen concentration ≥ ;

medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to **0.19.**

Note: PR (Platte River State Fish Hatchery), WL (Wolf Lake State Fish Hatchery), TH (Thompson **State Fish** Hatchery).

Fish strain	Gamete Donors		Fingerlings tested 8 month following egg-take		
	Year	*Renibacterium salmoninarum* prevalence	Year	Hatchery	*Renibacterium salmoninarum* prevalence
Swan River Chinook	2002	45.7 % Total 5.0 % high 1.7 % medium 39.0 % low	2003	PR	92.0 % Total 0.0 % high 50.0 % medium 42.0 % low
				TH	0.0 % Total 0.0 % high 0.0 % medium 0.0 % low
	2003	35.3 % Total 2.9 % high 0.0 % medium 32.3 % low	2004	PR	0 % Total 0.0 % high 0.0 % medium 0.0 % low
				WL	35.0 % Total 3.3 % high 0.0 % medium 31.7 % low
				TH	0.0 % Total 0.0 % high 0.0 % medium 0.0 % low
	2004	13.7 % Total 4.1 % high 1.6 % medium 8.0 % low	2005	PR	8.3 % Total 0.0 % high 0.0 % medium 8.3 % low
				WL	10.0 % Total 0 % high 1.7 % medium 8.3 % low
				TH	2.0 % Total 0.0 % high 0.0 % medium 2.0 % low

Table 16. *Renibacterium salmoninarum* infection prevalence and intensity among the Swan River Weir chinook salmon feral spawners and their corresponding hatchery raised fingerlings throughout the period from 2001 to 2005.

Data in this table was generated using a polyclonal antibody-based quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was determined by % of Q-ELISA positive samples of the total number of samples tested. Intensity was considered high if the antigen concentration ≥ ;

medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.

Note: PR (Platte River State Fish Hatchery), WL (Wolf Lake State Fish Hatchery), TH (Thompson State Fish Hatchery).
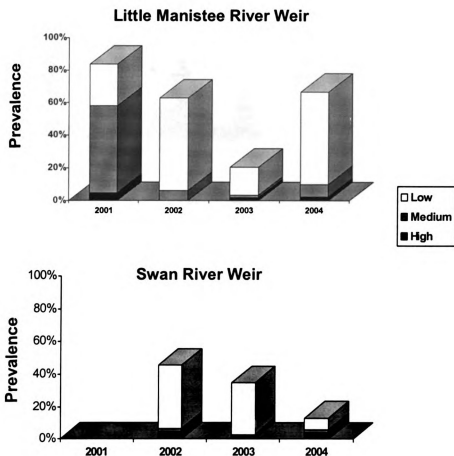
Figure 7. Prevalence of *Renibacterium salmoninarum* in feral chinook

salmon spawners collected from two of Michigan weirs between 2001 and

2005.

Data in this Figure was generated using a polyclonal antibody-based quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was determined by % of Q-ELISA positive samples of the total number of samples tested. Intensity was considered high if the antigen concentration ≥1; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.
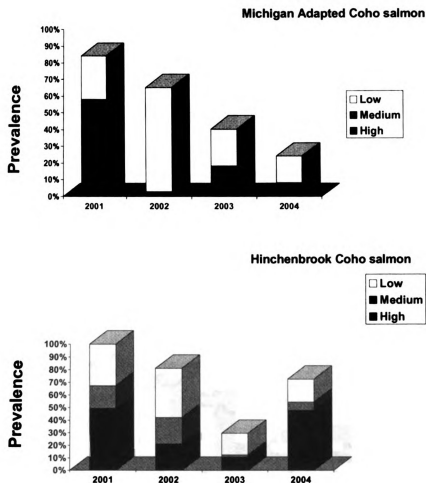
Figure 8. *Renibacterium salmoninarum* prevalences and intensities in feral

spawner coho salmon strains returning to Platte River Weir throughout

2001-2004

Data in this Figure was generated using a polyclonal antibody-based quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was determined by % of Q-ELISA positive samples of the total number of samples tested. Intensity was considered high if the antigen concentration ≥1; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.

Figure 9. Prevalence of *Renibacterium salmoninarum* in feral chinook salmon spawner males and females collected from two of Michigan weirs between 2001 and 2005.

Data in this Figure was generated using a polyclonal antibody-based quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was determined by % of Q-ELISA positive samples of the total number of samples tested. Intensity was considered high if the antigen concentration ≥ 1; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.
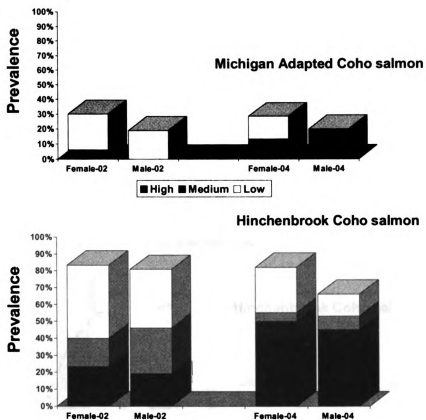
Figure 10. *Renibacterium salmoninarum* prevalence and intensity in

females and males of coho salmon spawners returning to Platte River Weir

throughout the period from 2002-2004.

Data in this Figure was generated using a polyclonal antibody-based quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was determined by % of Q-ELISA positive samples of the total number of samples tested. Intensity was considered high if the antigen concentration ≥1; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.

Michigan Adapted Coho salmon

Hinchenbrook Coho salmon
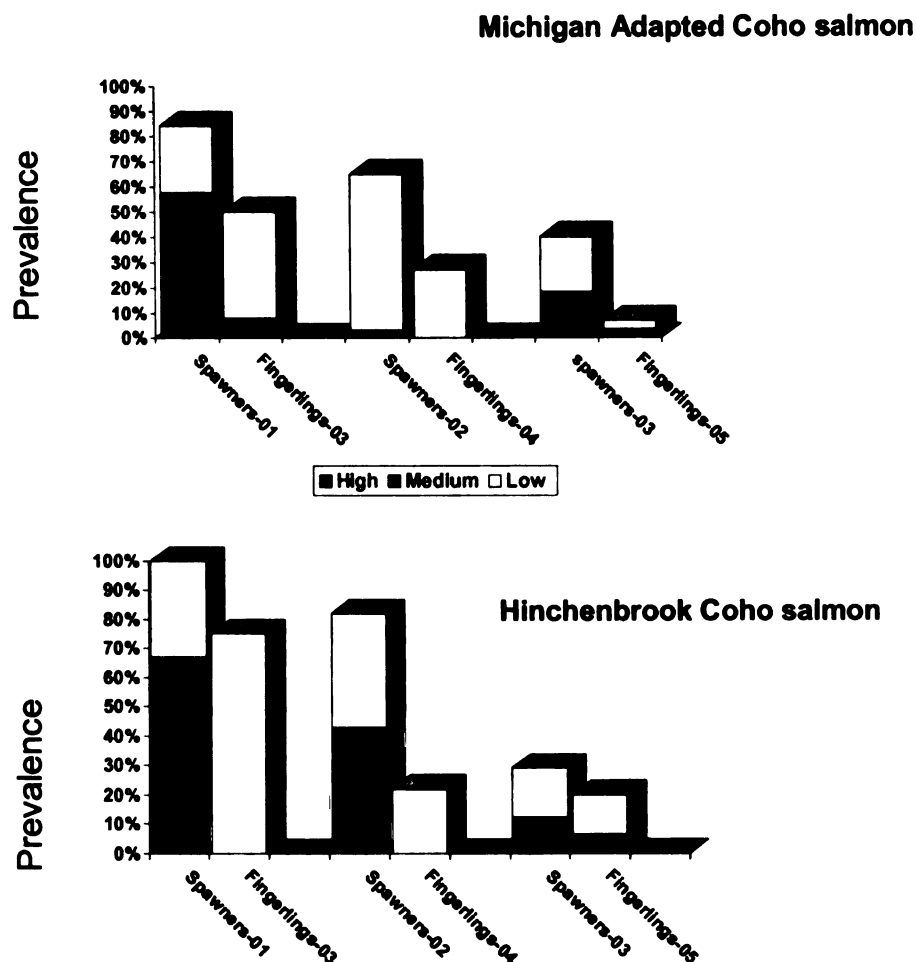
Figure 11. *Renibacterium salmoninarum* **prevalence and intensity of infection in gamete donor feral spawner coho salmon strains versus their corresponding 18-month juvenile fingerlings throughout 2001-2004.**

Data in this Figure was generated using a polyclonal antibody-based quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was determined by % of Q-ELISA positive samples of the total number of samples tested. Intensity was considered high if the antigen concentration ≥ 1; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.

CHAPTER FIVE

PREVALENCE, SHEDDING AND SPREAD OF *RENIBACTERIUM SALMONINARUM* IN BROOK TROUT (*SALVELINUS FONTINALIS*) WITH SPECIAL EMPHASIS ON THE ASSOCIATED DISEASE EPIZOOTICS IN MICHIGAN.

ABSTRACT

In order to determine the status of BKD in hatchery and wild populations of brook trout in Michigan, (*Renibacterium salmoninarum*) *R. salmoninarum* prevalence and intensity were determined in representative samples from adult hatchery raised and wild stocks as well as their offsprings from 2001 through 2005. The hatchery raised adult Iron River brook trout presented higher BKD prevalence than the wild Cherry Creek strain. Generally, the BKD prevalence and intensity in hatchery and wild brook trout strains gradually decreased throughout the period from 2001 to 2004. The critical role played by hatchery practices to control the spread and minimize the prevalence of BKD among Michigan brook trout populations was discussed. Although most of the previous studies reported an insignificant male role in transmission of *Renibacterium salmoninarum* (*R. salmoninarum*), yet our results clearly demonstrated that males shed along with their gametes more *R. salmoninarum* than females.

Number of BKD outbreaks among hatchery-reared brook trout populations has increased dramatically throughout the period of late spring of 2003 to the

early fall of 2004. The affected brook trout populations have been assessed using various diagnostic tools, such as clinical examination, Q-ELISA, culture, histopathology, and immunohistochemistry. The possible causes that lead to the initiation and progression of such outbreaks have been fully investigated. Although Q-ELISA, nested PCR, and culture results for all of the examined cases were positive, some of these cases were either barely within detection limits or demonstrated negative results in histopathological and immunohistochemical examination. This result is not surprising, as some of the specimens were preserved in formalin for a relatively long period (i.e. enough to affect the IHC power) even after performing improved antigen retrieval methods. Also, representative samples of 3-year-old broodstock exhibiting chronic forms of the disease had meager amounts of antigen remaining within their system, with trace amounts being present in the antigen antibody complexes in granulomas that occupied large portions of the kidney tissues. These were sequentially presented as a very light antigen score using the IHC (1+).

INTRODUCTION

Brook trout (*Salvelinus fontinalis*) is an indigenous salmonid species in the Great Lakes (Coon 1999) that has been artificially propagated and stocked in Michigan's public waters for years (Dexter and O'Neal, 2004). Two strains of brook trout, the Assinica and Iron River strain, are being used by used by Michigan Department of Natural Resources (MDNR) for supplementing resident stream populations where there is a deficiency of natural recruitment (Dexter and O'Neal, 2004). Assinica and Iron River brook trout are the two main strains of brook trout that are being reared and stocked in Michigan. Assinica brook trout are characterized by better survival and growth than domestic stocks (Gowing 1986). Iron River Brook trout are considered a pure native strain that is originally from the Iron River in Michigan's Upper Peninsula (Driver 1995). Unlike the Assinica strain, Iron River brook trout are slow to reach maturity and are characterized by a very slow growth rate because of their wild characteristics (Dexter and O'Neal 2004).

Fish health poses major challenges to development and progress of the brook trout restoration in the Great Lakes basin. Among these health challenges, Bacterial Kidney Disease, caused by *Renibacterium salmoninarum* (*R. salmoninarum*), is an eminent threat due to the enzootic nature of the pathogen within Great Lakes waters (Eissa 2005-Chapter 3). Moreover, affinity of *R. salmoninarum* for the kidneys, which possesses an essential lymphoid function and its obligate intracellular nature, makes this pathogen and its soluble antigens

a major threat to the host by suppressing the fish immune system (Ellis 1999; Fredriksen et al. 1997; Grayson et al. 2002).

A considerable number of studies have been performed on brook trout in the USA which indicated that brook trout is the most susceptible salmonid to BKD (Belding and Merrill 1935; Snieszko and Griffin 1955). However, most of these studies involved the use of experimental infection, while a relatively few were concerned with natural BKD infection (Allison et al. 1958; Mitchum et al. 1979; Mitchum and Sherman 1981). For example, Bullock et al. (1971); Mitchum and Sherman (1981) reported that brook trout mortalities from BKD are higher than that of the brown trout.

Interestingly, the first report of BKD in the USA occurred in brook trout at a Massachusetts State fish hatchery (Belding and Merrill, 1935). During the late 1940s and early 1950s, *R. salmoninarum* infection caused mass mortalities in brook trout at the federal hatcheries in Berlin, New Hampshire, Cortland, and New York (Snieszko and Griffin, 1955). Mitchum et al. (1979) determined the prevalence of BKD in dead and live brook trout collected from a small lake and stream system in southeastern Wyoming, USA, where BKD epizootics had been observed since 1972. They found that prevalence among dead fish and live fish at upstream stations was 100% and 83%, respectively.

In Michigan, the first case of BKD was discovered in 1955 in brook trout yearlings at the Oden and Marquette state hatcheries, where eggs were originally imported from a hatchery in New England in which BKD had been endemic for many years (Allison, 1958). Since the first report of the disease in 1955, none of

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the published studies have reported any data about the recent occurrence of BKD outbreaks, prevalence, or magnitude of the disease in brook trout in Michigan or in the Great Lakes basin.

Thus, the aim of the current research was to investigate the status and magnitude of BKD in brook trout in Michigan by assessing the *R. salmoninarum* prevalence in the hatchery raised brook trout populations in Michigan. The role-played by the male and the shedding of the bacteria along the gamete are also investigated. Moreover, a number of recently erupted BKD outbreaks among brook trout populations in Michigan were also investigated.

MATERIALS AND METHODS

Fish. To investigate the prevalence and intensity of *R. salmoninarum* infection among brook trout (BKT) populations in Michigan waters, a total of 628 adult brook trout were collected from the hatchery raceways of Marquette State fish hatchery (MSFH) and the Cherry Creek water stream outside the hatchery in Marquette, Michigan, from 2001-2004. MSFH is the primary facility for brook trout broodstock that are used for the production of fingerlings to be stocked in both inland and Great Lakes waters.

Kidney tissue samples were collected from a total of 567 hatchery-raised brook trout broodstocks (529 Iron River strain (IR-BKT) sample, 38 Assinica strain (AS-BKT) sample) and 61 adult Cherry Creek wild brook trout (CC-BKT) (Table17). Following approximately 18 months of egg incubation and fish rearing in the Marquette State Fish Hatchery, MDNR releases fingerlings in the spring of each year. Between 2002-2005, a total of 420 kidney tissues of pre-stocking fingerlings were collected and analyzed for the presence of *R. salmoninarum* prior to release into the basins of Lakes Michigan (table 17).

The sacrifice of broodstock was accomplished by exposing the fish to an overdose of MS-222 (tricaine methane sulfonate, Finquel- Argent Chemical Laboratories, Redmond, WA). Following gamete collection, the abdominal cavity was cut open to examine individual internal organs for signs of BKD, followed by collecting about one gram of tissues from anterior, posterior and middle kidney

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sections. Attention was given not to cross contaminate samples and dissecting tools were replaced with sterile ones following dissection of each fish. Fingerlings were sacrificed by immersion in an overdose of MS-222. In order to ensure that the sample is representative, as much tissue as possible was harvested from each kidney using sterile dissection tools. A total of 200 coelomic fluid samples were collected using sterile transfer pipette from the egg/ovarian fluid mixture, transferred to 5 ml sterile polypropylene tubes, and kept on ice until processed at the laboratory at Michigan State University, East Lansing, MI. Also, a total of 200 semen samples were collected directly from the middle stream of semen into sterile 10-ml polypropylene tubes. Fingerlings were sacrificed by immersion in an overdose of anesthesia using MS-222 (tricaine methane sulfonate, Finquel- Ardent Chemical Laboratories, Washington).

A number of BKD outbreaks of involving brook trout were recorded between 2003 and 2004. In late May of 2003, a total of 72 Iron River brook trout fingerlings with a recent history of mortalities were submitted to the Aquatic Animal Health Laboratory (AAHL) at Michigan State University from the Marquette State Fish Hatchery (MSFH) in Michigan. A course of treatment was prescribed and then followed by the collection of another 60 samples for post treatment analysis. In Mid July 2004, a total of 9 adult three year-old Assinica brook trout were submitted to the AAHL from the MSFH during an onset of broodstock losses. In Mid September of 2004, a total of 30 brook trout yearlings were submitted to the AAHL from Cedarbrook Fish Farm in Harrisville, Michigan that were experiencing increased mortalities.

Clinical examination. Fish were euthanized using an overdose of MS 222 (tricaine methane sulfonate) (Finquel- Argent Chemical Laboratories, Washington). Fish were then externally examined for the presence of any lesions, parasites, or abnormal growths on the skin or gills. Fish were dissected and examined internally for any lesions, swelling, or color changes in the kidneys other internal organs and viscera.

Sample processing. Kidney samples representing the anterior, posterior and middle sections of the kidney were transferred in sterile 7.5 cm x 18.5 cm Whirl Pak® bags (Nasco, Forte Atkinson, and WI), kept on ice, and were softened as much as possible through multiple cycles of physical pressure. The homogenized kidney tissues were diluted in 1:4 (w/v) Hank's Balanced Salt Solution (HBSS, Sigma Chemical Co, St. Louis, MO) then stomached for 2 minutes at high-speed in a Biomaster Stomacher-80 (Wolf Laboratories Limited, Pocklington, York, UK). In the case of ovarian fluid, 1 ml from fluid sample was diluted 1:2 (v/v) in HBSS for Q-ELISA testing. In the case of milt samples, 1ml from each semen sample was diluted 1:2 (v/v) in HBSS for Q-ELISA testing.

Measurements of *Renibacterium salmoninarum* antigen using the Quantitative Enzyme-linked Immunosorbent Assay (Q-ELISA). Aliquots of 250 µl volume of each samples were transferred into 1.5 ml safe-lock microfuge tube to which an equal volume of 0.01 M phosphate buffered saline-Tween 20

(0.05 %) (PBS-T20) (Sigma) with 5 % natural goat serum (Sigma) (Olea et al., 1993) and 50 μl CitriSolv solution (Fisher Chemicals, Fairlawn, New Jersey) (Gudmunsdottir et al., 1993) were added. The mixture were thoroughly mixed by vortexing then incubated at 100 °C on heat blocks with rotary shaker for 15 minutes and followed by 2 hours of incubation at 4 °C. After incubation, the mixture was centrifuged at 6000g for 15 minutes at 4 °C. The aqueous supernatant of each sample was carefully collected and then transferred to a 1.5 ml microfuge tube for Q-ELISA testing. The Q-ELISA method used in this study is that described in details by Pascho and Mulcahy (1987) and Alcorn and Pascho (2000). The positive negative threshold absorbances are calculated according to the method described by Meyers et al. (1993) .The positive–negative cutoff absorbance for the kidney homogenate was 0.10. The tested positive samples were assigned the following antigen level categories: low (0.10 to 0.19), medium (0.20-0.99) and high (1.000 or more) (Pascho et al., 1998). Intensity of infection among certain group of fish is expressed by percent of fish with high titers of *R. salmoninarum* soluble antigens using Q-ELISA.

Nested PCR. A DNeasy tissue extraction kit (Qiagen-Valencia, CA, USA) was used for the extraction of DNA from 100μl aliquots of kidney tissue homogenates. The DNA was extracted according to the manufacturer's instructions, with a few minor modifications from the method described by Pascho et al. (1998). The tissue pellets were obtained by centrifugation at 6000 g for 20 minutes at 4 °C and the pellets were incubated with lysozyme buffer

consisting of 180 µl of 20 mg lysozyme (Sigma), 20mM Tris-HCl, pH 8.0; 2 mM

EDTA (Sigma) and 1.2 % (v/v) Triton X 100 (Sigma) at 37 °C for 1 hour. The

nPCR method and primers recommended by Pascho et al. (1998) were

employed with slight modifications to the volume of DNA (5 µl for first round and

2 µl for second round PCR reaction), water, and master mixes (45 µl for first

round and 48 µl for second round nPCR reaction) .The controls were composed

of a PCR mixture containing no DNA template reagent (negative control), positive

R. salmoninarum and positive tissue control. A volume of 10 µl of the nPCR

product and controls were mixed with 2 µl of 6X loading dye (Sigma) and used on

a 2 % agarose gel (Invitrogen Life Technologies, Carlsbad, CA). Each

electrophoresis gel included a 1kbp DNA ladder with 100 bp increments

(Invitrogen). Gels were run in 1 X Tris Acetate Buffer (1 X TAE) gel buffer

(Sigma). Gels were visualized under the KODAK EDAS Camera System and UV

Trans-illuminator. Samples were considered positive when a 320 bp band was

detected. Molecular confirmation of the purified bacterial isolates was also

conducted using nPCR according to the method described by Chase and Pascho

(1998).

Histopathology. Kidney tissues fixed in 10 % neutral buffered formalin

solution were processed and embedded in paraffin. Five-micron sections were

routinely stained with Hematoxylin and Eosin (HE) using the method previously

described by Prophet et al. (1992). Slides were evaluated and given a score of 0,

+1, +2, or +3 based on degree of pathological alterations.

Immunohistochemistry (IHC). The methods used in the IHC were adopted

from Jansson et al. (1991) and Evensen et al. (1994). In brief, tissue sections of

kidney from BKT with clinical signs were used for immunohistochemical

evaluation of the expression *R. salmoninarum* soluble antigens. IHC staining was

performed on automated immuno-stainers. Paraffin embedded tissues that had

been fixed in formalin for less than 48 hours were deparaffinized in xylene,

rehydrated in graded ethanol, and rinsed in distilled water. Endogenous

peroxidases were neutralized with 3% hydrogen peroxide for 5 minutes. Antigen

retrieval was achieved by incubating slides in a citric buffer antigen retrieval

solution in a steamer (Black & Decker, Towson, MD) for 20 min, and non-specific

immunoglobulin binding was blocked by incubation of slides for 10 min with a

protein-blocking agent (Dako, Carpinteria, CA). Using the Dako autostainer

(Dako, Carpenteria, CA), slides were incubated for 30 minutes with a goat anti-

Renibacterium salmoninarum antibody (Kirkegaard & Perry Laboratories) at a

dilution of 1:100. A streptavidin-immunoperoxidase staining procedure (Dako,

Carpinteria, CA) was used for immunolabeling. The immunoreaction was

visualized with AEC (Dako, Carpinteria, CA). Sections were counterstained with

Mayer's hematoxylin. Tissues that had been fixed in formalin for more than a

year were immunostained using the protocol described above as well as the

following method to enhance antigen retrieval. In this particular protocol

deparaffinization, antigen retrieval, and immunostaining of formalin-fixed paraffin

embedded tissues were performed on the Bench Mark Automated Staining

System (Ventana Medical Systems, Inc.) using the Enhanced V-Red Detection (Alk. Phos. Red) Detection System (Ventana Medical Systems, Inc.) and a goat anti-*Renibacterium salmoninarum* antibody (Kirkegaard & Perry Laboratories) at a dilution of 1:100. Antigen retrieval was achieved using the Ventana Medical Systems Retrieval Solution CC1 (Ventana Medical Systems) for 60 min followed by digestion with and Protease 3 for 4min (Ventana Medical Systems). Sections were counterstained with haematoxylin. Positive IHC controls included a kidney from a trout with strong *Renibacterium salmoninarum* soluble antigens expression to which the appropriate antisera were added. For negative controls the primary antibodies were replaced with homologous non-immune sera.

Statistical Analysis. Because of the nature of data collected in this chapter required basic statistical description, data analysis was primarily relied on descriptive statistics. For year to year and brook trout strains comparisons, the data was tested for normality and then student t test (parametric) or Mann-Whitney Rank Sum Test was (with an alpha level = 0.05).

RESULTS

A. Prevalence of *R. salmoninarum* infection in captive and wild brook trout stocks. *Renibacterium salmoninarum* antigens were detected in the kidneys of Marquette State Fish Hatchery captive broodstock and fingerlings as well as Cherry Creek brook trout. The prevalence of *R. salmoninarum* in the Iron River broodstock exhibited a steady decline from 87% in 2001, to 80% in 2002, to 60% in 2003, and to 43% in 2004. Similarly, the intensity of *R. salmoninarum* in the Iron River brook trout (expressed by the percent of fish showing high titer of *R. salmoninarum* antigen) exhibited a comparable decline. The percent of fish with high *R. salmoninarum* antigen levels decreased from 17% in 2001 to 7.5% in 2004 (Figure 12).

Assinica brook trout Broodstock (BS AS-BKT) were tested only in 2003 and 2004. The results demonstrated a decrease in prevalence from 80 % in 2003 to 25% in 2004 (table 18). However, the intensity of the *R. salmoninarum* infection in the BS AS-BKT did not exhibit a similar decline.

In the case of Cherry Creek brook trout (CC-BKT), samples were collected in the falls of 2001-2004. Prevalence of *R. salmoninarum* in CC-BKT decreased from 80% in 2001 to 67% in 2003. The intensity of *R. salmoninarum* showed no consistent pattern (table 18).

The prevalence of *R. salmoninarum* in IR-BKT fingerlings between 2003 and 2005 were comparable to the parents and consistent with the high prevalence in

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the gamete donor broodstock, although 2004 showed a significantly low prevalence. However, the intensity of *R. salmoninarum* antigens in BKT fingerlings was higher than those detected in their parents during 2003 (48% vs 17% in their parents) before sharply declining in subsequent years (table 18).

The prevalence of *R. salmoninarum* in 2005-AS-BKT fingerlings was lower (28%) than those of their parent BS AS-BKT-2003. Similarly, the prevalence of *R. salmoninarum* in 2005-AS-BKT fingerlings was much lower than that of the 2005-IR-BKT fingerlings (28% vs 45% in IR-BKT fingerlings). The intensity of the *R. salmoninarum* infection in the AS-BKT fingerlings showed a decline, which was similar to the prevalence through the time of testing.

To compare the prevalence and intensity of *R. salmoninarum* in males and females BKT, two hundred pairs of IR-BKT broodstock were tested in 2004. Results indicated that the prevalence of *R. salmoninarum* infection was higher in the kidney tissue of the females than males (48.5% in females vs 37.5% in males). Similarly, the intensity of *R. salmoninarum* infection was clearly higher in the females (11% in female vs. 4 % in male). The shedding of the bacterial antigen along with the gametes was tested in broodstock IR-BKT in 2004. Data shown in Figure (13) illustrated that *R. salmoninarum* antigen shed with the gametes in both males and females IR-BKT. The prevalence of individuals that shed the bacterial antigen along with the gametes is lower in males than in females (10% in milt vs 15% in ovarian fluid). Also, the intensity of samples with high *R. salmoninarum* antigen was much higher in females (2.5%) than in males (0.5%). The majority of the females (20 out of 30) that shed the antigen in their

ovarian fluids exhibited similar levels of *R. salmoninarum* antigens in their

kidneys. However, 9 females shed the *R. salmoninarum* antigen along with the

ovarian fluid without detecting *R. salmoninarum* antigen in the kidneys and one

female shed the antigen at a low level in the ovarian fluid and tested negative for

the kidney tissue. Likewise, the majority of male shedders (13 out of 20) had

similar levels of *R. salmoninarum* in their kidneys with only 7 fish shed the

antigen without detected titer of *R. salmoninarum* antigen in the kidney.

B. BKD outbreaks among hatchery-raised brook trout. A number of BKD

outbreaks erupted among hatchery-raised brook trout during 2003 and 2004:

1. Iron River Brook trout. A major BKD outbreak has been observed in May,

2003. In this outbreak mortalities reached up to 50%. Externally, all fish

submitted for clinical examination had heavy infestations of skin monogenean

trematodes, sessile ciliates and *Trichodina* spp. Internally, the majority of fish

exhibited typical signs of BKD, such as enlarged kidneys with whitish gray

discoloration and the presence of multiple creamy-whitish nodules scattered

throughout the kidney tissues (Figure. 14). Kidney samples were tested for *R.*

salmoninarum using the nPCR, Q-ELISA and culture. Results indicated that *R.*

salmoninarum was present in all samples using each of the three techniques. All

tested fish had high concentration of *R. salmoninarum* antigens in the kidney

tissue by Q-ELISA. Examination of the histopathological slides of affected

kidneys revealed the presence of typical granulomatous reactions with a necrotic

center that was surrounded by a fibrous capsule, with a mixture of epithelioid macrophages, lymphocytes, and frequent giant cells. Gram-positive coccobacilli were observed within the necrotic tissue as well as other layers of the granuloma. When immunohistochemical (IHC) procedures were performed on the paraffin-waxed kidney sections, high positive results (scored 3+) where bacterial cells taking the dark red IHC staining were heavily distributed within the blue background of the kidney tissues (Figure 15 & 16). The Iron River brook trout fingerlings in the hatchery raceways were treated with 2.25% Aquamycin-100 in food. The fish were fed Erythromycin between 75 and 150 mg/kg of fish weight to comply with Investigational New Animal Drug (INAD) specifications and mortalities gradually decreased until subsiding completely at the completion of treatment. In early June of 2003, a total number of 60 erythromycin treated IR-BKT fingerlings were further examined post treatment. Results showed 4 of 60 fish (6.7 %) still had clinical BKD lesions. Kidneys of the examined fish were tested for the presence of *R. salmoninarum* antigens using Q-ELISA. Results indicated that *R. salmoninarum* antigens had sharply declined to 18.3 %, with only 5 % demonstrating the high titer of the antigens. Culture from post treatment fish showed that only 4 out of 60 fish (6.7 %) were positive.

2. Assinica Brook trout. In mid July of 2004, mortalities in the 3 year old Assinica brook trout stocks in MSFH continued for a month before representative samples were submitted for investigation. Random samples from fish in affected raceways were externally examined, where skin scraping revealed heavy infestations with monogenean (*Gyrodactylus* sp.). A total of 9 clinically affected

fish were examined, where 7 out of 9 fish (77.8 %) exhibited heavy monogenetic trematodes, sessile ciliates and fungal hyphae. Internally, 5 out 9 fish (55.6 %) exhibited grayish discoloration of the kidneys, with the presence of white abscess-like nodules scattered throughout the kidneys. Kidney samples from the 9 fish were further tested using nPCR, Q-ELISA and culture. All samples were positive when tested using nPCR, while a total number of 7 out of 9 (77.8 %) fish were culture positive and 3 out of 9 fish (33.3 %) were Q- ELISA positive (1 high, 1 medium, 1 low). When submitted for histopathology, 75 % of the kidney samples exhibited moderate multi-focal granulomatous reactions (scored 1+) and 25 % exhibited severe multi-focal granulomatous reactions (Scored 3+) (Figure 18). The reactions were characterized by the presence of typical granulomas, accompanied by a mixture of epithelioid macrophages, lymphocytes and occasional giant cells. Interestingly, the granulomatous reaction replaced 50-60 % of the renal parenchyma (Figure 18). Centrally, the lesion was composed of eosinophilic, caseous debris, surrounded by marked sheets, nests, and laminated foci of epithelioid macrophages mixed with lymphocytes. The periphery of the lesion was surrounded by mature fibrous connective tissue associated with more destruction of the renal parenchyma (Figure 17). Despite the fact that most of the samples showed the presence of typical granulomatous inflammation with varying severity (1+ to 3+ scores), the results of the IHC technique performed on the paraffin-waxed lesions were negative (Figure 18). The only sample with a typical granulomatous reaction exhibited very few bacterial cells when the

antigen retrieval technique was performed on the paraffin embedded blocks of the same samples (Figure 19).

3. Private Brook Trout Farm. In Mid September of 2004, mass mortalities occurred in the brook trout yearlings of a private Brook Trout Farm. Mortalities continued for two weeks. External parasitological examination of fish gills and skin revealed the presence of large numbers of monogenean trematodes and sessile ciliates. Internally, 12 of 30 fish (40 %) showed pale gray, whitish discolorations and swelling of the kidneys, with multiple abscess-like nodules scattered all over the kidneys. Typical colonies of *R. salmoninarum* were observed after 2 weeks on MKDM from a total of 18 out of 30 fish (60 %), which was later confirmed with nPCR. Q-ELISA showed that 18 out of 30 fish (60%) were positive for *R. salmoninarum* antigens, with an intensity of 67% (expressed by percent of fish with high titers of *R. salmoninarum* soluble antigens using Q-ELISA).

DISCUSSION

For decades, brook trout has been known for its high susceptibility to *R. salmoninarum* infection (Snieszko and Griffin 1955; Mitchum and Sherman 1981). Brook trout infected with *R. salmoninarum* either naturally or experimentally, suffer from high mortalities (Belding and Merrill 1935; Snieszko and Griffin 1955; Mitchum et al. 1979).

Data obtained in this study demonstrated a high prevalence and intensity of *R. salmoninarum* infection in both hatchery raised and wild stocks. This concurs with previous reports. For example, Mitchum and Sherman, 1981, recorded a relatively high prevalence and severity of *R. salmoninarum* infection in wild and hatchery raised brook trout populations (58 %, 45 % respectively).

A general trend of declining prevalence and intensity of *R. salmoninarum* in IR-BKT broodstock was observed over the period of this study. This trend might be explained by the improvement of hatchery hygienic practices. Among these practices are the prophylactic erythromycin phosphate administration and hardening of eggs in erythromycin containing water. Evelyn et al. 1986 and Lee and Evelyn (1989) found that intramuscular injection of broodstock with erythromycin phosphate dramatically minimized the vertical transmission of *R. salmoninarum*. Also, Rinsing of broodstock in iodophores solution before collecting gametes could minimize the *R. salmoninarum* on the eggshell (Ross and Smith 1972). Maule et al. (1996)

described similar observations that lead to remarkable decrease of *R. salmoninarum* prevalence among other salmonid species.

Although the Assinica strain demonstrated inconsistent *R. salmoninarum* infection prevalence when compared to the Iron River, yet Iron River strain showed higher intensity than Assinica strain. The Assinica strain is known for its superior survival and growth (Gowing 1986), and these characteristics could play a vital role in the general defense of the fish against severe infections with *R. salmoninarum*. In addition, variable susceptibility of fish strains to different diseases is not unusual. For example, some strains of steelhead showed variable susceptibility to *R. salmoninarum* (Winter et al., 1980).

Wild populations of BKT (CC-BKT) showed a comparable prevalence to that of the hatchery reared BKT. The fact that Cherry Creek supplies the hatchery with water and that BKT exists in this water may explain the similarity in infection levels.

Analysis of the data of *R. salmoninarum* prevalence and intensity of infection among the Iron River brook trout pre-stocking fingerlings indicated that fingerlings from 2003 exhibited the highest prevalence and infection intensity (approaching 100 %). The 2003 pre-stocking fingerlings are the offspring of the 2001 Iron River brook trout parent stocks that also exhibited high BKD prevalence (83 %). Vertical transmission have probably played a major role in this high incidence of infection, particularly that erythromycin prophylactic administration was not implemented in 2001. On the contrary,

the 2004-2005 Iron River and Assinica brook trout offspring showed relatively lower *R. salmoninarum* prevalence and intensity, although they originally hatched from fertilized eggs collected from 2002-2003 parents with relatively high *R. salmoninarum* prevalence and intensity. This could only be explained by the strict hygienic measures adopted by the hatchery starting from 2002.

Data obtained from the Q-ELISA testing of gametes indicated that the *R. salmoninarum* was shed with the gametes in both males and females (10% in males versus 15% in females), with a higher intensity in females than males. These results suggest a contribution of the male in the vertical transmission of *R. salmoninarum* to the offspring, in addition to the role played by females. Allison (1955) was the first to report vertical transmission in the brook trout, albeit with circumstantial evidence that gametes from infected adults resulted in infected offspring. Our data agreed with Wiens and Kaattari (1989), which were able to detect the *R. salmoninarum* antigens in the milt of infected males. However, the studies of Klontz (1983) and Evelyn, et al. (1986) demonstrated that males play an insignificant role in the vertical transmission of *R. salmoninarum*. However, the data may be complicated by discrepancies between levels of *R. salmoninarum* in the ovarian fluid and their levels in the kidney of corresponding individual fish.

BKD outbreaks were associated with severe clinical signs and high mortalities in BKT in Michigan. The frequent occurrence of BKD epizootics in hatchery raised brook trout populations during 2003 and 2004 without affecting other salmonids, such as the lake trout residing in the same

hatchery, presumptively indicates that brook trout has higher susceptibility to *R. salmoninarum* infection when compared to other char and trout species. Although brook trout (*Salvelinus fontinalis*) are known for their high susceptibility to *R. salmoninarum* infection (Snieszko and Griffin 1955; Mitchum and Sherman, 1981), reports of the disease in this species were scarce in Michigan. Data indicated that the clinical picture and the magnitude of the mortalities described in the current study coincide with previous reports about BKD-associated epizootics in brook trout in the United States (Belding and Merrill 1935; Snieszko and Griffin 1955; Mitchum et al. 1979).

Outbreaks described in the current study were frequently coupled with external parasitic infestations. It is not clear whether the external parasitic infestation initiated or resulted from the BKD outbreaks. However, it seems that many factors were involved in influencing mortalities associated with the outbreaks. First, the rise in water temperature, along with the heavy density in the hatchery raceways, favors the external parasites (Mo 1997; Rintamaki and Voltonen 1994). Heavy external parasitic infestations open portals of entry within the skin and gills of fish and facilitate the horizontal transmission of the *R. salmoninarum* in water via the skin and gills. It is well documented that the bacteria can survive in water and feces for up to 21 days (Austin and Rodger 1980s; Balfry et al. 1996). Also, a number of authors have hypothesized that water and feces indirectly act as reservoir for the transmission of *R. salmoninarum* from infected fish to other fish (Bullock 1975; Mitchum and Sherman 1981). Alternatively, the gradual increase of

water temperature during the time of the outbreaks was assumed by some authors to initiate the progression of BKD infection (Belding and Merrill 1935; Snieszko and Griffin 1955; Smith 1964; Sanders et al. 1978). The sequence of BKD infections is usually associated with the production of *R. salmoninarum* soluble antigens, which are known to suppress the immune response of infected fish (Fredriksen et al. 1997; Turaga et al. 1987; Wiens and Kaattari 1991, Ellis 1999), which drive the host to become vulnerable to infection with other bacteria and parasites.

The development of the disease in the 2003-2004 outbreaks was documented using both histopathology and IHC. Examination of kidney sections stained with H&E indicated the presence of severe granulomatous reactions, with typical granuloma components where the organism could be detected both intra-cellularly and extra-cellularly using the Gram stain. Previous records of histopathology support these pathologic pictures of the kidney lesions during both experimental and natural BKD infections (Wood and Yasutake 1956; Young and Chapman 1978; Bruno 1986; Sami et al. 1992). In case of the Iron River BKT mortalities, IHC stained slides indicated the presence of an intense distribution of bacterial cells and their antigens within the kidney tissues, as well as within the granulomas, which confirms the assumption that the severe progression of the disease is associated with high bacterial metabolic activities (Evenden et al.1993, Bruno 1986, Sami et al.1992). Similar findings were reached by Hoffman et al. (1989); Jansson et al. (1991); Evensen et al. (1994) and Lorenzen et al. (1996).

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Some discrepancies were observed between histopathological findings (positive with different scores) and IHC results (negative results of some of positive histopathological section). The histopathological findings ranged from mild multi-focal histiocytic inflammation (scored 1+), which appeared negative using the IHC stains to severe granulomatous reaction replacing more than 60 % of the kidney parenchyma (scored 3+). These kidney sections were negative when tested using regular IHC procedures and turned into very mild positive after improved antigen retrieval procedures were performed. Histopathological and IHC findings confirm the assumptions that the milder irritation over longer period of time might resulted in typical granuloma in one case, mild inflammation in others and negative results in the rest.

The Assinica BKT kidney samples were preserved in buffered formalin solution for more than 1 year which is reported to be crucial factor in reducing the sensitivity of IHC techniques for many antigens and epitopes (Sompuram et al., 2004). This long time preservation in buffered formalin solution could resulted in denaturation of the soluble antigens (p57 protein) and formation of Methylene bridges, both inter- and intra-molecularly which will ultimately alter the physical characteristics of the kidney tissues and also induces masking of the antigens (p57) (Evensen et al., 1994). These deleterious effects of formalin might be a good cause for the inability to detect the bacterial cells within the affected kidney tissues using IHC, although they were positive with other techniques like Q-ELISA, culture, and nPCR. An enzymatic digestion and heat treatment methods (antigen retrieval methods) (Evensen et al.,

1994) were used intensively to retrieve the masked antigens from the IHC processed tissues. Unfortunately the antigen retrieval methods were only able to retrieve extremely few *R. salmoninarum* soluble antigens within a typical granulomatous reaction of kidney tissue. One important disadvantage of the antigen retrieval method using heat treatment is the unexpected dissociation of the major soluble antigen (p57), which is known to be heat labile (Evensen et al.1994).

In conclusion, this study supports the previous reports, which emphasized that brook trout are highly susceptible to *R. salmoninarum* infection. In addition, this study shed the light on the possible contribution of a number of factors to development of BKD epizootics in Michigan hatcheries, such as the seasonal changes and the presence of external parasites. Further, the possible role of males and females in shedding the bacterium and its soluble antigens was fully discussed.

Brook trout strain and life stage	Date Collected	Number of fish tested
Iron River broodstocks	October, 2001	54
	October, 2002	45
	October, 2003	30
	October, 2004	400
Assinica broodstocks	October, 2003	30
	October, 2004	8
Iron River 18 month old pre-stocking fingerlings	March, 2002	60
	March, 2003	60
	March, 2004	60
	March, 2005	60
Assinica 18 month old pre-stocking fingerlings	March, 2003	60
	March, 2004	60
	March, 2005	60

Table 17. Details of samples collected from brook trout broodstocks throughout the period from 2001-2004 and pre-stocking 18-month-old fingerlings collected throughout the period from 2002-2005.

Strain	Parent stocks (Gamete donors)		18 months old pre-stocking fingerlings	
	Year	*Renibacterium salmoninarum* prevalence	Year	*Renibacterium salmoninarum* prevalence
Iron River	2001	87.0 % Total 16.7% high 50.0 % medium 20.4% low	2003	98.0 % Total 48.0 % high 35.4 % medium 14.6 % low
Iron River	2002	80.0 % Total 15.55 % high 13.3 % medium 51.1 % low	2004	20.0 % Total 0.0 % high 10.0 % medium 10.0 % low
Iron River	2003	60.0 % Total 10.0 % high 0.0 % medium 50.0 % low	2005	45.0 % Total 0.0 % high 12.5 % medium 32.5 % low
Assinica	2003	80.0 % Total 0.0 % high 10.0 % medium 70.0 % low	2005	27.5 % Total 0.0 % high 2.5 % medium 25.0 % low

Table18. *Renibacterium salmoninarum* infection prevalence and intensity among brook trout broodstocks and their corresponding 18 months pre-stocking fingerlings throughout the period from 2001 - 2005.

Data in this table was generated using a polyclonal antibody-based quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was determined by % of Q-ELISA positive samples of the total number of samples tested. Intensity was considered high if the antigen concentration ≥ 1; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.
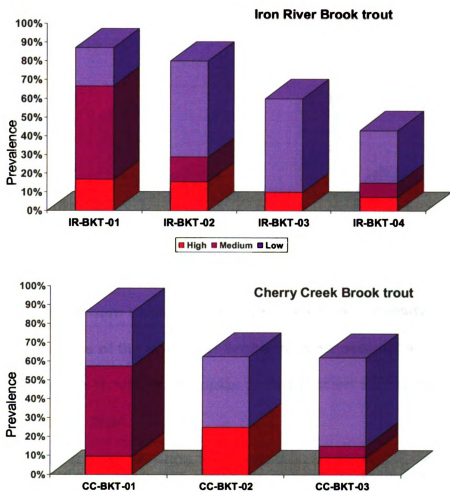
Figure 12. Prevalence and intensity of *Renibacterium salmoninarum*

among the adult brook trout collected through 2001-2003

Data in this Figure was generated using a polyclonal antibody-based
quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was
determined by % of Q-ELISA positive samples of the total number of samples
tested. Intensity was considered high if the antigen concentration ≥ 1; medium
if the antigen concentration = 0.20-0.99; and Low if the antigen
concentration = 0.10 to 0.19.
IR-BKT: Iron River Brook trout
CC-BKT: Cherry Creek Brook trout

This figure is in color
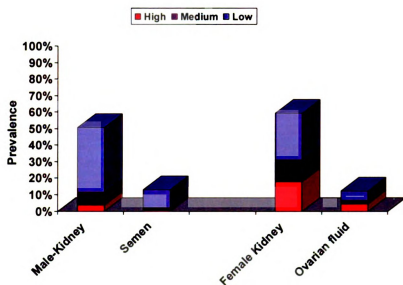
Figure 13. *Renibacterium salmoninarum* **Prevalence and intensity in**

kidneys and gametes of the Iron River brook trout broodstock in from

Marquette State Fish Hatchery. Samples were collected during the fall

spawning season of 2004.

Data in this Figure was generated using a polyclonal antibody-based quantitative ELISA (Q-ELISA) performed on kidney tissues. Prevalence was determined by % of Q-ELISA positive samples of the total number of samples tested. Intensity was considered high if the antigen concentration ≥1; medium if the antigen concentration = 0.20-0.99; and Low if the antigen concentration = 0.10 to 0.19.

This Figure is in color
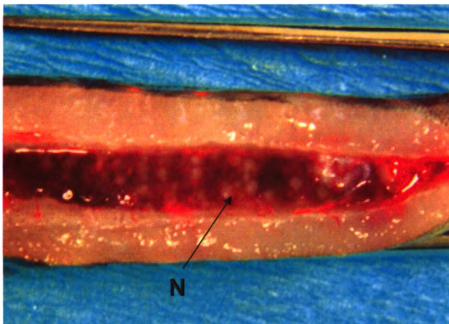
Figure 14. An Iron River Brook trout fingerling with Bacterial Kidney Disease. The kidney is swollen with multiple creamy-whitish nodules (N).

The above case is from an outbreak of Bacterial Kidney Disease that killed thousands of hatchery raised Iron River brook trout fingerlings in May 2003.

This Figure is in Color
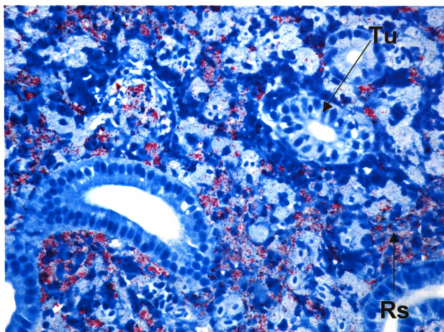
Figure15. Kidney tissue of Iron River brook trout fingerling exhibiting heavy *Renibacterium salmoninarum* infection. Kidney section was stained using an anti-*Renibacterium salmoninarum* antibody based streptavidin -immunoperoxidase immmunolbeling (Magnification 400). Sections were counterstained with Mayer's hematoxylin (Blue background)

Rs: *Renibacterium salmoninarum* soluble antigens with the red staining affinity.
Tu: Non-affected kidney tubules with blue counterstaining affinity.

This figure is in color
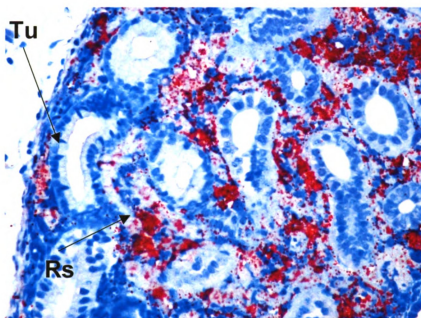
Figure 16. Kidney tissue of Iron River brook trout fingerling exhibiting heavy *Renibacterium salmoninarum* infection after enhanced antigen retrieval procedures using Alkaline Phosphatase Red and goat anti-*Renibacterium salmoninarum* antibody. Sections were counterstained with Mayer's Hematoxylin (Blue background) (Magnification 400). This case is from an outbreak of BKD that killed thousands of hatchery raised Iron River brook trout fingerlings in May 2003.

Rs: *Renibacterium salmoninarum* soluble antigens with the red staining affinity.
Tu: Non-affected kidney tubules with blue counterstaining affinity. The marked increase in dark red dots (retrieved bacteria) distribution within the kidney tissue after the antigen retrieval compared to the picture in figure 16.

This figure is in color
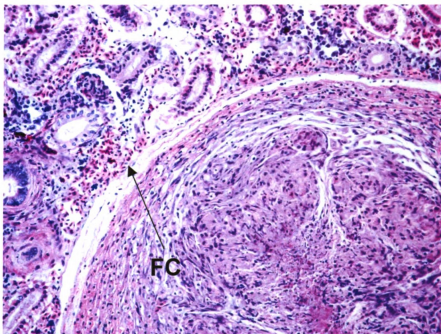
Figure 17. Hematoxylin and Eosin stained slide of kidney showing a severe granulomatous reaction that is replacing kidney tissues of a 3 years old Assinica brook trout. Notice the fibrous capsule (FC) surrounding the entire granuloma (Magnification 100). The case is from an outbreak of BKD that killed captive 3 years old Assinica brook trout in mid September 2003.

This figure is in color
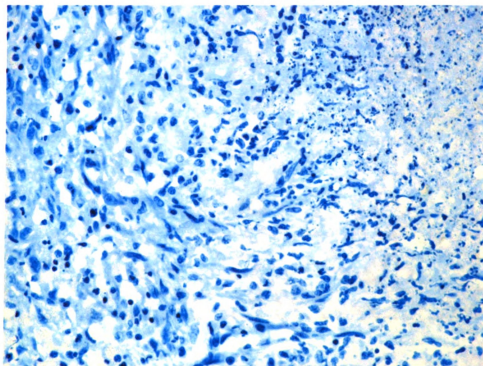
Figure 18. An immunohistochemical stained Kidney tissue section with chronic multi-focal granulomatous reaction from a 3 years old Assinica brook trout with absence of bacterial cells or antigen from the affected kidney tissues. Kidney section was stained using an anti-*Renibacterium salmoninarum* antibody based streptavidin - immunoperoxidase immmunolbeling (Magnification 400). Sections were counterstained with Mayer's hematoxylin (Blue background). The case is from an outbreak of BKD that killed captive 3 years old Assinica brook trout in mid September 2003.

This figure is in color
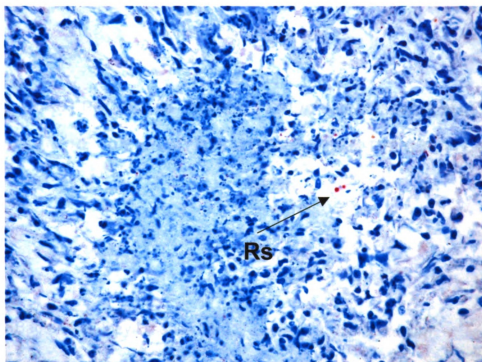
Figure 19. An immunohistochemical stained Kidney tissue section with chronic multi-focal granulomatous reaction from a 3 years old Assinica brook trout exhibiting a mild chronic presence of bacterial cells or antigens after enhanced antigen retrieval procedures using Alkaline Phosphatase Red and goat anti-*Renibacterium salmoninarum* antibody. Sections were counterstained with Mayer's Hematoxylin (Blue background) (Magnification 400).

Rs: *Renibacterium salmoninarum* soluble antigens with the red staining affinity.

This figure is in color

CHAPTER SIX

FIRST RECORD OF *RENIBACTERIUM SALMONINARUM* IN SEA LAMPREY (*PETROMYZON MARINUS*) FROM THE GREAT LAKES BASIN

ABSTRACT

Bacterial Kidney Disease (BKD), caused by *Renibacterium salmoninarum* (*R. salmoninarum*), is a widespread problem with major implications for salmonid fish. The mechanisms by which the bacteria have reached high levels of infection previously unrecorded in the Laurentian Great Lakes are presently unknown. Research involving reservoirs and mechanisms of *R. salmoninarum* transmission in fish is lacking due to the ecological complexity of heterogeneous habitats and the lack of adequate funding. Surprisingly, we isolated *R. salmoninarum* from the kidneys of the sea lamprey (*Petromyzon marinus*). The bacterium was cultured from kidneys of 16% and 4% of Lake Ontario lampreys examined in 2003 and 2004 respectively, with bacterial colonies verified via nested polymerase chain reaction (nPCR) and quantitative enzyme linked immunosorbent assay (Q-ELISA).

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INTRODUCTION

Bacterial Kidney Disease (BKD), caused by *Renibacterium salmoninarum*, (*R. salmoninarum*) is a serious bacterial disease of salmonines (Fryer and Sanders, 1981) that is widespread in the Great Lakes basin (Hnath and Faisal 2005). Many facts pertaining to disease transmission and reservoirs of infection are currently unknown. The majority of studies conducted thus far suggest that *R. salmoninarum* exclusively infect salmonids and that carrier fish are responsible for its distribution (Wood and Yasutake 1956; Klontz 1983; Bullock and Herman 1988). However a few studies have indicated that non-salmonid fish species such as Pacific hake (*Merluccius productus*, Kent et al. 1998) and Pacific herring (*Clupea harengus pallasi*, Paclibare et al. 1988) may harbor the pathogen. In addition, certain fish, species such as Pacific herring (Traxler and Bell 1988), Sablefish (*Anoplopoma fumbria*) (Bell et al. 1990), common shiners (*Notropis cornutus*) (Hicks et al. 1986) and fathead minnows (*Pimephales promelas*) (Hicks et al. 1986) contracted the infection when exposed to *R. salmoninarum* via intraperitoneal injection. The role that non-salmonid species may play in the spread of BKD has not been investigated in the Great Lakes basin.

In the Great Lakes basin, a number of non-indigenous species have invaded the system and caused serious ecologic and economic losses (Lupi

and Hoehn 1998). Among these invasive species, is the sea lamprey (*Petromyzon marinus*), which has been one of the most destructive of the introduced species. The sea lamprey has been incriminated as a major factor contributing to the collapse of the lake trout (*Salvelinus namaycush*) and the lake whitefish (*Coregonus clupeaformis*) fisheries in the Great Lakes during the 1940s and 1950s from which these two fisheries have yet to fully recover despite the advent of sea lamprey chemical control since 1958 (Smith and Tibbles 1980).

To further reduce the number of sea lamprey and limit its spread, the Great Lakes Fishery Commission began a large-scale experimental program based on trapping male sea lamprey, sterilizing them, and subsequently releasing the sterile males back into streams where they compete with fertile males for spawning females. Field assessments indicated a decreased hatch rate in streams where this strategy was practiced. Up to 40,000 sterilized sea lamprey are released annually, yielding major success in the areas of implementation, such as the St. Marys River. Currently, males are collected from different areas in the Great Lakes basin, transported into a sterilizing facility in Hammond Bay, Michigan, and then released into selected river systems basin-wide. These transfers of lamprey to different locations may additionally transfer various pathogens concurrently, the probability of which has raised major concerns regarding the possibility of resident fish populations becoming infected.

To this end, this current study was initiated to determine if the sea lamprey could be a new host range for *R. salmoninarum* infection and the possible vector role that may contribute to the spread of *Renibacterium salmoninarum*.

MATERIALS AND METHODS

Sea Lamprey (*Petromyzon marinus*). In the midsummer of 2003, 25 adult sea lamprey were moved from the Humber River and Duffins Creek, Lake Ontario, and presented alive to the Aquatic Animal Health Laboratory (AAHL) at Michigan State University to undergo fish health inspection and thereby ascertain their suitability for transfer to the lamprey sterilizing facility at Hammond Bay, then into the St. Mary's River, both within the Lake Huron watershed. In midsummer 2004, an additional 118 adult lamprey were caught from Duffins Creek and Humber River, held separately, and brought alive to the AAHL. Until examined, lampreys were maintained alive in well-aerated chilled aquaria.

Dissection, sampling and sample processing. Lampreys were euthanized with an overdose of MS 222 (tricaine methane sulfonate, Finquel-Ardent Chemical Laboratories, Washington) and dissected under aseptic conditions. Kidneys were removed aseptically and placed in sterile 7.5 cm x 18.5 cm Whirl-Pak® bags (Nasco, Fort Atkinson, WI) to which Hank's Balanced Salt Solution (HBSS) was then added at a ratio of 1:4 (weight/ volume) and stomached for 120 seconds using a high-speed Biomaster Stomacher-80 (Wolf Laboratories Limited, Pocklington, York, UK).

Bacterial isolation and identification. A100 µl aliquots of stomached kidney tissue were spread onto MKDM (Eissa 2005, Chapter 2). Inoculated plates were incubated for up to 20 days at a sub-ambient temperature incubator adjusted to 15 °C. Incubated plates were checked for colonial growth on daily basis. Isolates were identified according to the standard morphological and biochemical criteria for *R. salmoninarum* as described by Sanders and Fryer (1980), Austin and Austin (1999) and Bruno and Munro (1986). Biochemical tests included: motility, in motility test medium (BD and Company Sparks, MD, USA), cytochrome oxidase determination on Pathotec strips (Remel, Lenexa, Kansas, USA), catalase with 3 % hydrogen peroxide, esculin hydrolysis using bile esculin agar (Remel), and a DNAse test using DNAse test medium (Remel). Carbohydrate utilization was performed using basal oxidation fermentation media (DIFCO-BD) that was prepared according to manufacturer instructions prior to the addition of individual sugars. Aseptically, 10 ml of filter sterilized (0.45 µm) 10 % sugar solution, was added to autoclaved and cooled (48 °C) basal media to reach a final concentration of 1 % with the exception of salicin, which was made as a 5 % solution to reach a 0.5 % final concentration. Each of the following sugars was added individually to the basal medium arabinose, glucose, lactose, maltose, rhamnose, salicin, sucrose, sorbitol, xylose. All sugars were from Sigma.

Nested PCR. A DNeasy tissue extraction kit (Qiagen-Valencia, CA,

USA) was used to extract DNA from 100μl aliquots of kidney tissue

homogenates. The DNA was extracted according to manufacturer's

instructions, with a few minor modifications from the method described by

Pascho et al. (1998). Tissue pellets were obtained by centrifugation at 6000

g for 20 minutes at 4 °C and the pellets were incubated with lysozyme buffer

consisting of 180 μl of 20 mg lysozyme (Sigma), 20mM Tris-HCl, pH 8.0; 2

mM EDTA (Sigma) and 1.2 % (v/v) Triton X 100 (Sigma) at 37 °C for 1 hour.

The nPCR method and primers recommended by Pascho et al. (1998) were

employed with slight modifications to the volume of DNA (5 μl for first round

and 2 μl for second round PCR reaction), water, and master mixes (45 μl for

first round and 48 μl for second round nPCR reaction) .The controls were

composed of a PCR mixture containing no DNA template reagent (negative

control), positive *R. salmoninarum* and positive tissue control. A volume of 10

μl of the nPCR product and controls were mixed with 2 μl of 6X loading dye

(Sigma) and used on a 2 % agarose gel (Invitrogen Life Technologies,

Carlsbad, CA). Each electrophoresis gel included a 1kbp DNA ladder with

100 bp increments (Invitrogen). Gels were run in 1 X Tris Acetate Buffer (1 X

TAE) gel buffer (Sigma). Gels were visualized under the KODAK EDAS

Camera System and UV Trans-illuminator. Samples were considered

positive when a 320 bp band was detected. Molecular confirmation of the

purified bacterial isolates was also conducted using nPCR according to the

method described by Chase and Pascho (1998).

Q-ELISA. The sample preparation and Q-ELISA protocol was adopted

from the methods detailed in Pascho and Mulcahy (1987) and Alcorn and

Pascho (2000). The positive negative threshold was determined according to

the calculations detailed in Meyers et al. (1993). The positive –negative cutoff

absorbance for the kidney homogenate was 0.10. The samples that tested-

positive were assigned the following antigen level categories: low (0.10 to

0.19), medium (0.20-0.99) and high (1.000 or more) (Pascho et al., 1998).

RESULTS

Isolation, identification and confirmation of *Renibacterium*

salmoninarum. *Renibacterium salmoninarum* isolates were retrieved from

the kidneys of 4 out of 25 (in 2003) and 5 out of 118 (in 2004) adult sea

lamprey from the Duffins Creek/Humber River assemblage. The organism

was not isolated from blood samples in 2003 nor 2004 (table 19).

Morphologically, all isolates were Gram-positive diplobacilli or coccobacilli.

On MKDM agar plates, the isolates produced 1 mm diameter, white, shiny,

smooth, round colonies with raised surfaces. In MKDM broth, most of the

isolates produced white granular pellets with the exception of two isolates

(SL 14 and SLHR 15) that produced uniform turbidity with large white pellets.

Biochemically, all the retrieved isolates were non-motile, catalase positive,

cytochrome oxidase negative, esculin hydrolysis negative and DNAse

negative. The bacteria did not produce acid from any of the carbohydrates

that were tested (table 20). These bacterial isolates were identified as *R.*

salmoninarum and they were confirmed using nPCR (Figure 20) and Q-

ELISA.

Detection of *R. salmoninarum* in sea lamprey kidneys. Bands of 320

bp, characteristic of *R. salmoninarum* were visualized using the nPCR

technique in four (16 %) out of 25 lamprey kidney samples collected in the

mid summer of 2003 (table 19). Using the same technique the organism was

detected in 38 (66 %) out of 58 lamprey kidney samples collected in 2004

from the Duffins Creek site (table 19), while positive nPCR was recorded in 3

(5 %) out of 60 lamprey kidney samples collected from the Humber River site that year. When the Q-ELISA technique was performed on the 2004 samples, *R. salmoninarum* antigens were detected in the kidneys of two (1.7 %) of the 118 lamprey and were low in titer (table 19).

DISCUSSION

Despite the fact that *R. salmoninarum* has been described as a salmonid-specific pathogen, it has been isolated for the first time from the sea lamprey (*Petromyzon marinus*) for two successive years (2003 and 2004). The prevalence in sea lamprey is relatively low when compared to the prevalence found in salmonines. Despite the isolation of *R. salmoninarum* from the kidneys of clinically affected sea lamprey, the bacterium was not isolated from the blood nor any other internal organs. It appears that sea lamprey *R. salmoninarum* isolates possess the same affinity for kidney tissues as that of salmonid isolates.

The morphological criteria and biochemical reactions of the retrieved sea lamprey isolates coincided with those described for *R. salmoninarum* (Sanders and Fryer 1980). The size of the detected amplicon band (320 bp) in both kidney tissues and cultured isolates using nPCR was consistent with that published for *R. salmoninarum* (Pascho et al., 1998; Chase and Pascho, 1998).

While all *R. salmoninarum* isolates obtained in this study were positive with both nPCR and Q-ELISA, tissues from which the isolates were retrieved were not always positive. This discrepancy could be attributed to the presence of tissue inhibitors, present in the sea lamprey that may interfere with PCR or ELISA reactions. As Makos and Youson (1988) reported, the sea lamprey does not have a gall bladder, thus bile salts accumulate in the

muscles and kidneys. Biochemically, bile salts act as a detergent, which may contribute, to the inhibition of diagnostic assays.

Inversely, in the case of Duffins Creek samples, other than SLDC6, nPCR yielded positive results while no *R. salmoninarum* was isolated. This finding could be explained by the presence of low numbers of bacteria that could be detected with nPCR, but are less than the threshold that allows their isolation. This threshold has been estimated by 100-colony forming units/gram tissue in salmonids fish (Lee 1989). The nested PCR assay can detect as little as 4-10 bacterial cells/gram tissue (Miriam, et al., 1997).

In summary, this study reports the sea lamprey as a host for *R. salmoninarum*. The role played by Great Lakes sea lamprey in the epizootiology of BKD in the Great Lakes requires further investigations.

Sea lamprey	Site of collection	Type of sample	% Positive Cultured Samples	% Positive nPCR samples	% Positive Q-ELISA samples
2003 SL1-25-03	Humber/Duffins Lake Ontario	Kidney	(4/25) 16 %	(4/25) 16 %	ND
2003 SL1-25-03	Humber/Duffins Lake Ontario	Blood	(0/25) 0 %	ND	ND
2004 SLHR1-60	Humber River Lake Ontario	Kidney	(4/60) 6.7 %	(3/60) 5 %	(2/60) 3.3 %
2004 SLHR1-60	Humber River Lake Ontarion	Blood	(0/60) 0 %	ND	ND
2004 SLDC1-58	Duffin's Creek Lake Ontario	Kidney	(1/58) 1.7 %	(38/58) 65.5 %	0 %
2004 SLDC1-58	Duffin's Creek Lake Ontario	Blood	(0/58) 0 %	ND	ND

Table 19. Prevalence of *Renibacterium salmoninarum* in kidneys and blood of 2003-2004 Lake Ontario sea lampreys using nPCR, Q-ELISA and culture.

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Isolate ID	Culture-based assays						nPCR	Q-ELISA
	C	O	E	D	CHO	M		
SL3-03	+	-	-	-	-	-	+	+
SL21-03	+	-	-	-	-	-	+	+
SL11-03	+	-	-	-	-	-	+	+
SL14-03	+	-	-	-	-	-	+	+
SLDC 6-04	+	-	-	-	-	-	+	+
SLHR 9-04	+	-	-	-	-	-	+	+
SLHR14-04	+	-	-	-	-	-	+	+
SLHR15-04	+	-	-	-	-	-	+	+
SLHR16-04	+	-	-	-	-	-	+	+

Table 20. Confirmation of *Renibacterium salmoninarum* isolates

using some culture based assays, serological and molecular assays.

Notice: SL3, SL21, SL11 and SL14 were the isolates retrieved from adult sea lamprey in mid summer 2003. DC6 was an isolate retrieved from adult sea lamprey number 6 collected from Duffins Creek, Lake Ontario. HR9, HR14, HR15 and HR16 were isolates retrieved from adult sea lamprey collected from Humber River, Lake Ontario. Both DC and HR isolate were retrieved from adult sea lamprey collected in the mid summer 2004. Culture based assays: C (catalase), O (oxidase), E (esculin hydrolysis), D (DNAse), CHO (carbohydrate utilization) and M (Motility). Serological assay: (Q-ELISA -Polyclonal antibody based quantitative ELISA). Molecular assay: (nPCR-Nested polymerase chain reaction)

Figure 20. Nested PCR assay performed on isolates showing the

characteristic 320 bp band of *Renibacterium salmoninarum* isolates.

Notice: SL3, SL21, SL11 and SL14 were the isolates retrieved from adult sea lamprey in mid summer 2003. DC6 was an isolate retrieved from adult sea lamprey number 6 collected from Duffins Creek, Lake Ontario. HR9, HR14, HR15 and HR16 were isolates retrieved from adult sea lamprey collected from Humber River, Lake Ontario. Both DC and HR isolate were retrieved from adult sea lamprey collected in the mid summer 2004. Samples were considered positive when a 320 bp band was detected.
L: 1kbp DNA ladder with 100 bp increments

CHAPTER SEVEN

CONCLUSIONS AND FUTURE RESEARCH

CONCLUSIONS

Although BKD exists in Michigan since 1955, only little is known about the status and magnitude of the disease in salmonids and water basin of Michigan. The only available information about the disease was from publications in Europe, Canada and Pacific Northwest. *Renibacterium salmoninarum*, the causative agent of BKD grows slowly *in vitro* and *in vivo* and incubation period might extend to up to 12 weeks before obtaining bacterial growth. Further, most of the published studies concerning these issues showed marked inconsistency. Bacterial Kidney Disease is focal in its distribution within the affected kidneys, thus targeting a part of the kidney other than the focus of infection (granulomas) might lead to negative testing results. All the aforementioned factors were the triggering factors that lead me to develop my research on BKD in Michigan salmonines.

Initially, in order to overcome the slow growing nature and the longer incubation period of the pathogen, an optimized tissue processing and culture procedures together with a modified growth medium have been developed. The protocol and modified medium have significantly shortened the incubation time and allowed culturing of *R. salmoninarum* with relative ease throughout the study. These achievements allowed the isolation of relatively large number of *R. salmoninarum* isolates from both cultured and wild salmonines. These isolates

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constitute a unique resource for future studies of *R. salmoninarum* and BKD in the Great Lakes. Findings also demonstrated that *R. salmoninarum* is widespread in Michigan.

Using the analysis of different diagnostic testing patterns produced from different agreements and disagreements in results of diagnostic BKD testing assays (Nested PCR, Quantitative ELISA, and Culture) I was able to track the progress of natural *R. salmoninarum* infection in some of Michigan's salmonid stocks. The disagreement in results among the three assays was linked to different phases of *R. salmoninarum* infection at the time of sampling. The testing results demonstrated the presence of diagnostic testing patterns, with each of the patterns representing a probable stage along the course of *R. salmoninarum* infection.

Data generated in chapter four of this dissertation provided additional evidence that *R. salmoninarum* infection is enzootic in Michigan watersheds. Lake Michigan's chinook salmon tend to have overall higher *R. salmoninarum* prevalence than that in Lake Huron's fish. Data also indicated that *R. salmoninarum* infection and intensity in returning feral salmon seem to fluctuate among years, although a definitive decrease can be observed since the start of this study in 2001. Data of this study demonstrated the ability of both chinook (*Oncorhynchus tshawytscha*) and coho (*Oncorhynchus kisutch*) females and males to shed *R. salmoninarum* along with gametes. The BKD testing of both feral spawners and their hatchery raised offspring fingerlings demonstrates that

the current testing and culling programs have been partially successful in reducing R. salmoninarum transmission.

In chapter five, a gradual decrease in R. salmoninarum prevalence in the hatchery raised and wild brook trout (salvelinus fontinalis) was noticed throughout the period from 2001 to 2004. The role-played by hatchery practices in minimizing the spread and prevalence of BKD among Michigan brook trout populations was discussed. Despite the fact that most of the epidemiological studies performed in the past diminished the role played by males in transmission of the R. salmoninarum, yet data presented in this study indicated that males can shed a fair amount of R. salmoninarum and its soluble antigens into the milt which increase the possibilities played by male in spreading the disease. Also, a number of BKD outbreaks involving the brook trout have been investigated using a number of diagnostic techniques through the duration of the study. Analysis of results presented in this chapter confirmed the previously reported assumption that brook trout is highly susceptible to R. salmoninarum infection.

Finally, data generated in chapter six indicated that the non salmonid adult sea lamprey is a new host range for R. salmoninarum and might possibly play a role in the dissemination of R. salmoninarum during the process of relocation of adult stages between number of watersheds in great lakes including Lake Michigan and Lake Huron watersheds.

FUTURE RESEARCH

The data obtained in this dissertation established a solid base for future research on *Renibacterium salmoninarum* (*R. salmoninarum*) infection in salmonids and non-salmonid species in Michigan. Further research is required to study the effect of each ingredient of the modified media on growth of the bacteria. Further, the chemical constituents of the *R. salmoninarum* metablites released in culture media or into in the infected tissue are needed to be identified in detail and wethere there is a dissimilarity or resemblance between *In vivo* and *In vitro* production of metabolites. Molecular basis behind the antibiotic resistence of some *R. salmoninarum* strains should be fully investigated.

Shedding of *R. salmoninarum* in male along with the milt has been proven in the current study. Yet, whether the bacteria are shed in the seminal fluid or passed on within the spermatozoal cells are needed to be elucidated.

Concurrent infections with *R. salmoninarum* and external parsites or other systemic bacteria as *Aeromonas salmonicida* and *Flavobacterium* sp. were noticed during the current study. The sequential mechanisms of such concurrent infections needed to be fully investigated and addressed.

Renibacterium salmoninarum was isolated for the first time from the kidneys of the adult parsitic seas lamprey. This work requires further research to determine the mechanism of infection in lampreys. Also, the possible role palyed by Sea lamprey in spreading the disease to salmonids needs to be illucidated.

Kidneys are the primary targets of *R. salmoninarum* (Fryer and Sanders, 1981); however, other organs should also be assessed in future studies to better understand BKD pathogenesis, particularly in natural infections.

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