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Authors: Faisal, M., and Eissa, A. E.

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DIAGNOSTIC TESTING PATTERNS OF *RENIBACTERIUM SALMONINARUM* IN SPAWNING SALMONID STOCKS IN MICHIGAN

M. Faisal[1,2,4] and A. E. Eissa[1,3]

[1] Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan 48824, USA
[2] Department of Fisheries and Wildlife, College of Agriculture and Natural Resources, Michigan State University, East Lansing, Michigan 48824, USA
[3] Department of Fish Medicine and Management, Faculty of Veterinary Medicine, Cairo University, Guiza, Egypt 12211
[4] Corresponding author (email: faisal@msu.edu)

ABSTRACT: Bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum*, is a slowly progressing disease that threatens salmon conservation and restoration programs in North America. The purpose of this study was to track naturally occurring *R. salmoninarum* infection in representative, Michigan, USA, 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 semiquantitative 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 natural *R. salmoninarum* infection. Findings also suggest that fish stocks tested in this study were not uniform in the distribution of the diagnostic patterns and that, from studying such patterns, one can determine the course of BKD infection in a particular population.

Key words: Bacterial kidney disease, diagnostic patterns, *Renibacterium salmoninarum*.

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. In addition to horizontal transmission, *R. salmoninarum* can be transmitted vertically (Evelyn et al., 1984, 1986a, 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 BKD 75 yr ago, 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 techniques, quantitative enzyme-linked immunosorbent assay (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). 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).

Inconsistencies among findings were often difficult to interpret because of methodologic limitations that entail sensitivity, specificity, specialized equipment, and labor-intensive procedures. For example, it has been estimated that the nPCR 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 bacteria/g kidney tissues (Pascho et al., 1998). Culture, on the other hand, requires the presence of 40–100 bacteria/g to ensure bacterial isolation (Lee, 1989; Miriam et al., 1997). The Q-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 1.3×10^4 bacteria/ml of ovarian fluid and 10^3

bacteria/g of kidney tissues are needed to produce consistently 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. It is well documented that *R. salmoninarum* can be transmitted vertically from parents to offspring (Evelyn et al., 1984, 1986a, b). 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; MacIntosh et al., 2000) and skin lesions (Evenden et al., 1993). Once the infection is established, *R. salmoninarum* secretes a number of soluble proteins that play a role in pathogenicity (Bruno, 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). *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 tissue before death (Bruno, 1986).

Most studies have concentrated on the discrepancies produced in the diagnostic tests to merely evaluate the sensitivities of various detection assays. Therefore, the aim of this study was to use the limits of detection for each assay and what each assay detects to evaluate the potential for diagnostic discrepancies. This information is needed to better understand the kinetics and course of natural infection within the Great Lakes salmonid fish stocks.

MATERIALS AND METHODS

Fish

A total of 364 feral and captive, spawning salmonids were collected from Michigan, USA, 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), USA, and from the Swan River Weir (SRW) at Rogers City, Presque Isle County, Michigan (Lake Huron watershed), USA. An additional 131 Michigan–adapted and 53 Hinchenbrook coho salmon (*Oncorhynchus kisutch*) were collected from the Platte River Weir (PRW) at Beulah, Michigan (Lake Michigan watershed), USA. 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 the Michigan, USA, Upper Peninsula. Males and females were equally represented among samples. Feral spawners were euthanized by exposing the fish to carbon dioxide–laden water. Following gamete collection, the abdominal cavity was cut open for the collection of kidney tissues.

Sampling and sample processing

Samples from fish were analyzed individually, unless otherwise indicated. Most of the kidney tissues encompassing the anterior, middle, and posterior sections of the kidney were collected in sterile 7.5×18.5-cm Whirl Pak® bags (Nasco, Fort Atkinson, Wisconsin, USA), kept on ice, and softened as much as possible through multiple cycles of physical pressure. To ensure the release of *R. salmoninarum* from granulomatous tissues and its even distribution in the sample, homogenized kidney tissues were diluted in 1:4 (w/v) Hank's Balanced Salt Solution (HBSS; Sigma Chemical Co, St. Louis, Missouri, USA) and then stomached for 2 min at high-speed using the Biomaster Stomacher-80 (Wolf Laboratories Limited, Pocklington, York, UK). Each stomached kidney tissue sample was then tested for the presence of *R. salmoninarum* or its soluble antigens using the following assays.

Culture: Aliquots of stomached kidney tissues were spread onto modified kidney disease medium (MKDM), which consists of 1% w/v peptone (Sigma), 0.05% w/v yeast extract (Sigma), 0.1% w/v L-cysteine hydrochloride (HCl; Sigma), and 0.005% w/v cycloheximide (Sigma) dissolved in distilled water. Following autoclaving at 121 C for 15 min, the medium was left to cool down to 48 C, and then newborn calf serum (Sigma, 10% v/v), 0.22-μm filter-sterilized *R. salmoninarum* spent broth (1% v/v), oxolinic acid (0.00025% w/v; Sigma), polymyxin B sulfate (0.0025% w/v;

Sigma), and D-cycloserine (0.00125% w/v; Sigma) were added. The medium's pH was adjusted to 6.8, and 1.5% w/v agar (Remel Inc., Lenexa, Kansas, USA) was added.

Inoculated plates were incubated for up to 20 days at 15 C and checked daily for bacterial growth. Identification of the isolates was done according to the standard morphologic and biochemistry criteria for *R. salmoninarum* (Sanders and Fryer, 1980; Austin and Austin, 1999). Molecular confirmation of the isolates was done using the nPCR method (Pascho et al., 1998).

Measurements of R. salmoninarum antigen using the Q-ELISA: The Q-ELISA method, described by Pascho and Mulcahy (1987) and Alcorn and Pascho (2000), has been followed, with some modifications. Aliquots of each sample (250 µl) were transferred into 1.5-ml Safe-Lock microfuge tubes, to which an equal volume of 0.01-M phosphate-buffered saline–Tween 20 (PBS-T20; 0.05%; Sigma), with 5% natural goat serum (Sigma), as recommended by Olea et al. (1993), and 50-µl CitriSolv solution (Fisher Chemicals, Fairlawn, New Jersey, USA), as recommended by Gudmondsdottir et al. (1993), were added. The solution was then thoroughly mixed, incubated at 100 C on heat blocks with a rotary shaker for 15 min, then incubated for 2 hr at 4 C. The mixture was centrifuged at 6,000 × G for 15 min at 4 C. The aqueous supernatant of each sample was used for the Q-ELISA testing. 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 (0.10–0.19), medium (0.20–0.99), and high (1.000 or more), as recommended by Meyers et al. (1993) and Pascho et al. (1998). Each assay included two negative controls (a negative fish tissue sample and a dilution buffer) and two positive controls (a positive tissue sample and the standards supplied with the kit).

Nested PCR: Bacterial DNA was extracted using the DNeasy tissue extraction kit (Qiagen, Valencia, California, 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 6,000 × G for 20 min at 4 C and were then incubated with lysozyme buffer consisting of 180 µl of 20 mg lysozyme (Sigma), 20 mM Tris-HCl (pH 8.0; Sigma), 2 mM ethylenediaminetetraacetic acid (EDTA; Sigma), and 1.2% (v/v) Triton ×100 (Sigma) at 37 C for 1 hr. The nPCR method used primers recommended by Pascho et al. (1998), with slight modifications to the volume of DNA (5 µl for the first round and 2 µl for the second round of nPCR) and master mixes (45 µl for the first round and 48 µl for the second round of 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 6× loading dye (Sigma) and used on a 2% agarose gel (Invitrogen Life Technologies, Carlsbad, California, USA). Each electrophoresis gel included a 1-kbp DNA ladder with 100-bp increments (Invitrogen). Gels were run in 1× Tris acetate–EDTA buffer (1× TAE; Sigma). Gels were visualized under the Kodak Enhanced Data Acquisition System (EDAS; Kodak, Rochester, New York, USA) camera and ultraviolet (UV) transilluminator (Kodak). Samples were considered positive when a 320-bp band was detected.

RESULTS

As displayed in Table 1, among the 364 fish examined, 343 fish (94.2%) were positive in the nPCR assay, 281 (77.2%) were positive in the culture method, and 246 (67.6%) were positive with the Q-ELISA method. Over half (53.3%) of the fish used in this study gave positive results by all three diagnostic assays. The consistency among findings was highest in the case of Hinchenbrook coho (81.1%), followed by brook trout (63.4%). Only seven fish (1.9%) were negative by all three diagnostic assays.

Most fish found positive by Q-ELISA possessed low *R. salmoninarum* antigen concentrations (193 of 231; 83.5%); the highest proportions of which, were in the LMRW Manistee chinook salmon and the PRW Michigan-adapted coho salmon (64.3 and 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

TABLE 1. Diagnostic testing patterns among salmonid feral spawners and captive broodstocks collected from Michigan, USA, in fall 2002. Data represented as number of positive fish (percentage of positive fish out of total tested).

Fish and source	No. fish tested	Diagnostic testing patterns,[a] No. (%)					
		1	2	3	4	5	6
Little Manistee River Weir Chinook Salmon	42	0 (0)	11 (26)	25 (60)	1 (2)	5 (12)	0 (0)
Swan River Weir Chinook Salmon	58	15 (26)	15 (26)	12 (21)	8 (14)	5 (9)	2 (3)
Platte River Weir Michigan-adapted Coho Salmon	131	3 (2.3)	34 (26)	75 (57.2)	18 (13.7)	0 (0)	2 (1.5)
Platte River Weir Hinchenbrook Coho Salmon	53	1 (2)	8 (15)	43 (81)	1 (2)	0 (0)	0 (0)
Marquette State Fish Hatchery Brook Trout	41	4 (10)	2 (5)	26 (63)	8 (20)	1 (2)	0 (0)
Marquette State Fish Hatchery Lake Trout	39	1 (3)	17 (44)	13 (31)	2 (5)	3 (8)	3 (8)
Total	364	24 (6.7)	87 (23.9)	194 (53.3)	38 (10.4)	14 (3.8)	7 (1.9)

[a] Pattern 1: Polymerase chain reaction positive (PCR+), enzyme-linked immunosorbent assay negative (ELISA−), and culture negative (culture−); Pattern 2: PCR+, ELISA−, and culture+; Pattern 3: PCR+, ELISA+, and culture+; Pattern 4: PCR+, ELISA+, and culture−; Pattern 5: PCR−, ELISA+, and culture−; and Pattern 6: PCR−, ELISA−, and culture−.

samples, six patterns were recognized. Pattern 1 represented fish that were positive with the nPCR only (24/364; 6.7%). Pattern 2 represented 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 represented 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 seven fish (1.9%) that had negative results with all three assays used in this study.

The distribution of fish representing each of the patterns gave a model that differed among fish stocks tested. For example, the majority of LMRW chinook salmon were in Patterns 2 and 3 (upper panel in Fig. 1), whereas SRW chinook salmon showed a wider distribution consisting of more than half of the fish in Patterns 1 and 2 (lower panel in Fig. 1). In the case of PRW Michigan-adapted coho, almost all fish were in Patterns 2–4 (upper panel in Fig. 2). On the other hand, 75% of the Hinchenbrook coho salmon strain belonged to Pattern 3 (lower panel in Fig. 2). Similarly, the two *Salvelinus* spp. captive broodstocks, although kept in the same hatchery, exhibited different diagnostic testing patterns (Figs. 3 and 4), with more than 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 seven fish were negative out of 364 when tested by the three diagnostic assays. Although these figures are staggering, one should not be surprised because *R. salmoninarum* has existed in Michigan salmonines for at least 50 yr (Allison, 1958) and was involved in the massive chinook salmon die offs of the 1980s in Lake Michigan (Holey, 1998). However, it should also be emphasized that fish tested in this study were spawning adults (>4 yr old), a factor that increases the likelihood of exposure to *R. salmoninarum* and allows time for the slow progression of

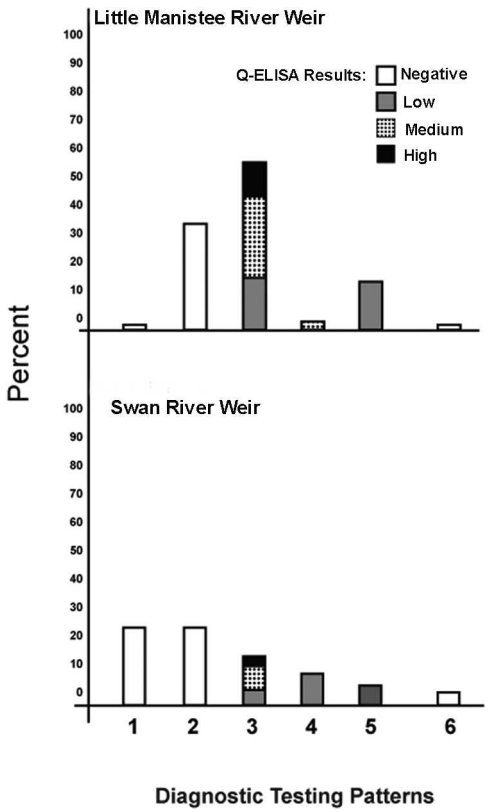

FIGURE 1. *Renibacterium salmoninarum* diagnostic testing patterns among spawning chinook salmon returning to either the Little Manistee River weir or the Swan River weir, Michigan, USA. Patterns 1–6 signify the following: Pattern 1: PCR+, ELISA−, culture−; Pattern 2: PCR+, ELISA−, culture+; Pattern 3: PCR+, ELISA+, culture+; Pattern 4: PCR+, ELISA+, culture−; Pattern 5: PCR−, ELISA+, culture−; and Pattern 6: PCR−, ELISA−, culture−. The bars represent patterns, and within each bar, Q-ELISA results are displayed as percentage of the total number of fish examined. PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; + = positive; − = negative; Q-ELISA = quantitative ELISA.

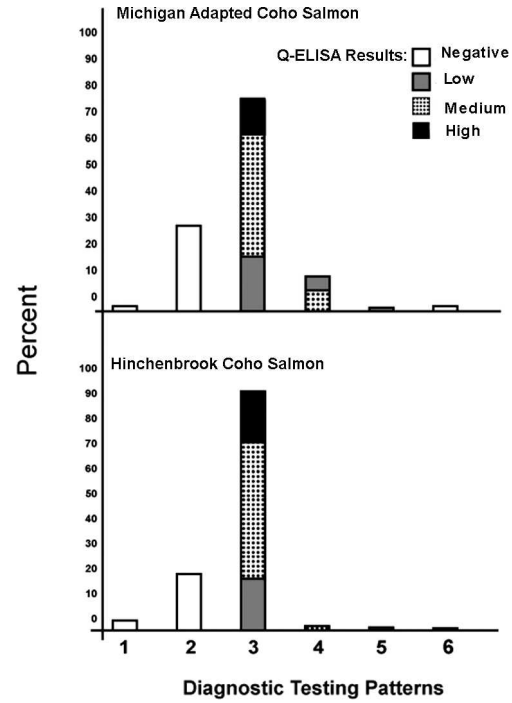

FIGURE 2. *Renibacterium salmoninarum* diagnostic testing patterns among spawning coho salmon (*Oncorhynchus kisutch*) returning to the Platte River weir. Patterns 1–6 signify the following: Pattern 1: PCR+, ELISA−, culture−; Pattern 2: PCR+, ELISA−, culture+; Pattern 3: PCR+, ELISA+, culture+; Pattern 4: PCR+, ELISA+, culture−; Pattern 5: PCR−, ELISA+, culture−; and Pattern 6: PCR−, ELISA−, culture−. The bars represent patterns, and within each bar, Q-ELISA results are displayed as percentage of the total number of fish examined. PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; + = positive; − = negative; Q-ELISA = quantitative ELISA.

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 because of 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 (*Salvelinus* spp.) species.

In other areas of the world where *R. salmoninarum* is enzootic, prevalence of infection in feral and wild fish species can attain the extremely high levels observed in this study. For example, in Iceland, arctic char (*Salvelinus alpinus*) and brown trout (*Salmo trutta*) reached infection levels of 100 and 81%, respectively (Jónsdóttir et al., 1998). In North America, *R. salmoninarum* infection rates of 83% in

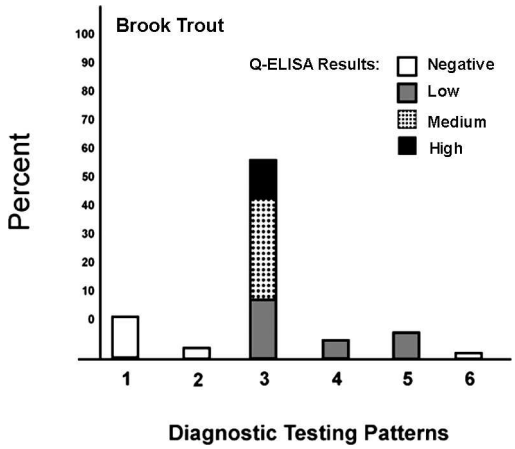

FIGURE 3. *Renibacterium salmoninarum* diagnostic testing patterns among captive brook trout broodstock kept at Marquette State Fish Hatchery. Patterns 1–6 signify the following: Pattern 1: PCR+, ELISA−, culture−; Pattern 2: PCR+, ELISA−, culture+; Pattern 3: PCR+, ELISA+, culture+; Pattern 4: PCR+, ELISA+, culture−; Pattern 5: PCR−, ELISA+, culture−; and Pattern 6: PCR−, ELISA−, culture−. The bars represent patterns, and within each bar, Q-ELISA results are displayed as percentage of the total number of fish examined. PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; + = positive; − = negative; Q-ELISA = quantitative ELISA.

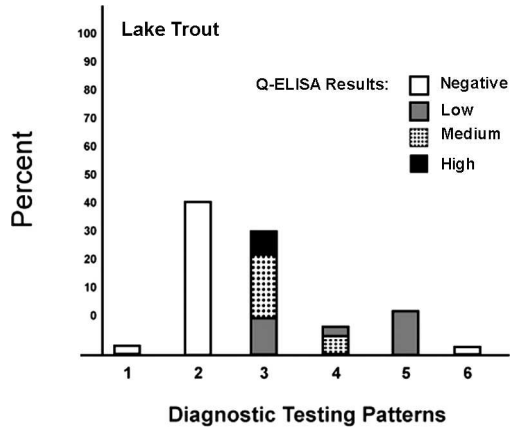

FIGURE 4. *Renibacterium salmoninarum* diagnostic testing patterns among captive lake trout broodstock kept at Marquette State Fish Hatchery. Patterns 1–6 signify the following: Pattern 1: PCR+, ELISA−, culture−; Pattern 2: PCR+, ELISA−, culture+; Pattern 3: PCR+, ELISA+, culture+; Pattern 4: PCR+, ELISA+, culture−; Pattern 5: PCR−, ELISA+, culture−; and Pattern 6: PCR−, ELISA−, culture−. The bars represent patterns, and within each bar, Q-ELISA results are displayed as percentage of the total number of fish examined. PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; + = positive; − = negative; Q-ELISA = quantitative ELISA.

brook trout from Wyoming, USA (Mitchum et al., 1979), and 35% in returning Atlantic salmon (*Salmo salar*) 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 increased 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).

Quantitative 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 with fish with higher *R. salmoninarum* antigen concentrations, a matter that could be associated with either early or late stages of infection (Sami et al., 1992; Jónsdóttir et al., 1996).

The lack of agreement in results of the three assays is difficult to explain but is not, however, surprising because 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 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. Although 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 pathologic manifestations of BKD, including granuloma formation (Miriam et al., 1997; Jónsdóttir et al., 1998). This is most likely because *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; 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 because 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 are deposited in the kidney glomeruli and are eliminated slowly through the kidneys (Sami, 1992). Fish in Pattern 6 were either never exposed to *R. salmoninarum*, were refractory to infection, or were infected and then totally eliminated *R. salmoninarum* and its antigen from their systems. Because *R. salmoninarum* is ubiquitous in Michigan, USA, 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. For example, most of the tested LMRW chinook salmon and PRW Michigan-adapted coho salmon (both returning from Lake Michigan) were either in Pattern 2 (>25%) or Pattern 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 of these stocks, overt clinical signs of BKD and mortalities (in the case of brook trout) are often observed (Faisal and Eissa, unpubl. obs.). Brook trout are known for their high susceptibility to *R. salmoninarum* infection (Snieszko and Griffin, 1955; Mitchum et al., 1979). This also explains the differences in *R. salmoninarum* infection patterns between the two *Salvelinus* spp. raised in the same hatchery. Hinchenbrook coho salmon strain was introduced to the Great Lakes basin from New York State relatively recently (Eisch, pers. comm.) and has proven to be more susceptible to *R. salmoninarum* infection when compared with the Michigan-adapted coho salmon strain that was introduced to the Great Lakes basin in 1967 (Hnath and Faisal, 2005).

Although 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 have relied upon experimental infection (White et al., 1995; Flaño et al., 1996). 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.

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LITERATURE CITED

ALCORN, S. W., AND R. J. PASCHO. 2000. Single-dilution enzyme-linked immunosorbent assay for quantification of antigen-specific salmonid antibody. Journal of Veterinary Diagnostic Investigation 12: 245–252.

ALLISON, L. N. 1958. Multiple sulfa therapy of kidney disease among brook trout. Progressive Fish Culturist 20: 66–68.

AUSTIN, B., AND D. A. AUSTIN. 1999. Bacterial fish pathogens: Diseases in farmed and wild fish, 3rd Edition. Springer-Praxis, Chichester, UK. pp. 17–18.

BALFRY, S. K., L. J. ALBRIGHT, AND T. P. T. EVELYN. 1996. Horizontal transfer of *Renibacterium salmoninarum* among farmed salmonids via the fecal-oral route. Diseases of Aquatic Organisms 25: 63–69.

BRUNO, D. W. 1986. Histopathology of bacterial kidney disease in laboratory infected rainbow trout, *Salmo gairdneri* Richardson, and Atlantic salmon, *Salmo salar* L., with reference to naturally infected fish. Journal of Fish Diseases 9: 523–537.

———, AND A. L. S. MUNRO. 1986. Haematological assessment of rainbow trout, *Salmo gairdneri* Richardson, and Atlantic Salmon, *Salmo salar* L., infected with *Renibacterium salmoninarum*. Journal of Fish Diseases 9: 195–204.

CHASE, D. M., AND R. J. PASCHO. 1998. Development of a nested polymerase chain reaction for amplification of a sequence of the p57 gene of *Renibacterium salmoninarum* that provides a highly sensitive method for detection of the bacterium in salmonid kidney. Diseases of Aquatic Organisms 34: 223–229.

CIPRIANO, R. C., C. E. STARLIPER, AND J. H. SCHACHTE. 1985. Comparative sensitivities of diagnostic procedures used to detect bacterial kidney disease in salmonid fishes. Journal of Wildlife Diseases 21: 144–148.

DENSMORE, C. L., S. A. SMITH, AND S. D. HOLLADAY. 1998. In vitro effects of the extracellular protein of *Renibacterium salmoninarum* on phagocyte function in brook trout (*Salvelinus fontinalis*). Veterinary Immunology Immunopathology 62: 349–357.

EVELYN, T. P. T., J. E. KETCHESON, AND L. PROSPERI-PORTA. 1984. Further evidence for the presence of *Renibacterium salmoninarum* in salmonid eggs and for the failure of providon-iodine to reduce the intra-ovum infection rate in the water. Journal of Fish Diseases 7: 173–182.

———, ———, AND ———. 1986a. Experimental intraovum infection of salmonid eggs with *Renibacterium salmoninarum* and vertical transmission of the pathogen with such eggs despite their treatment with erythromycin. Diseases of Aquatic Organisms 1: 197–202.

———, ———, AND ———. 1986b. Use of erythromycin as a means of preventing vertical transmission of *Renibacterium salmoninarum*. Diseases of Aquatic Organisms 2: 7–11.

EVENDEN, A. J., T. H. GRAYSON, M. L. GILPIN, AND C. B. MUNN. 1993. *Renibacterium salmoninarum* and bacterial kidney disease—The unfinished jigsaw. Annual Review of Fish Diseases 3: 87–104.

FAISAL, M., AND J. G. HNATH. 2005. Fish health and diseases issues in the Laurentian Great Lakes. *In* Health and diseases of aquatic organisms: Bilateral perspectives, R. C. Cipriano, I. S. Shchelkunov, and M. Faisal (eds.). Michigan State University, East Lansing, Michigan, pp. 331–350.

FLAÑO, E., S. L. KAATTARI, B. RAZQUIN, AND A. J. VILLENA. 1996. Histopathology of the thymus of coho salmon *Oncorhynchus kisutch* experimentally infected with *Renibacterium salmoninarum*. Diseases of Aquatic Organisms 26: 11–18.

FRYER, J. L., AND J. E. SANDERS. 1981. Bacterial kidney disease of salmonid fish. Annual Review of Microbiology 35: 273–298.

GRAYSON, T. H., L. F. COOPERS, AND A. B. WRATHMELL. 2002. Host responses to *Renibacterium salmoninarum* and specific components of the pathogen reveal the mechanisms of immune suppression and activation. Journal of Immunology 106: 273–283.

GUDMONDSDOTTIR, S., E. BENEDIKSDOTTIR, AND S. HELGASON. 1993. Detection of *Renibacterium salmoninarum* in salmonid kidney samples: a comparison of results using double sandwich ELISA and isolation on selective medium. Journal of Fish Diseases 16: 185–195.

HAMEL, O. 2001. The dynamic and effects of bacterial kidney disease in Snake River spring chinook salmon (*Oncorhynchus tshawytscha*). PhD. Dissertation, University of Washington, Washington D.C., 5446 pp.

HNATH, J., AND M. FAISAL. 2005. Bacterial kidney disease: Historical perspectives. Michigan Department of Natural Resources, Lansing, Michigan.

HOLEY, M. E., R. E. ELLIOT, S. V. MARCQUENESKI, J. G. HNATH, AND K. D. SMITH. 1998. Chinook salmon epizootics in Lake Michigan: Possible contributing factors and management implications. Journal of Aquatic Animal Health 10: 202–210.

JANSSON, E., T. HONGSLO, J. HOGLUND, AND O. LJUNGBERG. 1996. Comparative evaluation of bacterial culture and two ELISA techniques for the detection of *Renibacterium salmoninarum* antigens in salmonid kidney tissues. Diseases of Aquatic Organisms 27: 197–206.

JÓNSDÓTTIR, H., H. J. MACMQUIST, S. S. SNORRASON, G. GUDBERGSSONS, AND S. GUDMUNDSDOTTIR. 1998. Epidemiology of *Renibacterium salmoninarum* in wild Arctic charr and brown trout in Iceland. Journal of Fish Biology 53: 322–339.

LEE, E. G. H. 1989. Technique for enumeration of *Renibacterium salmoninarum* in fish kidney tissues. Journal of Aquatic Animal Health 1: 25–28.

MCINTOSH, D., B. AUSTIN, E. FLAÑO, J. A. VILLENA, M. P. TARAZONA, AND J. V. MARTINEZ-PEREDA. 2000. Lack of uptake of *Renibacterium salmoninarum* by gill epithelia of rainbow trout. Journal of Fish Biology 56: 1053–1061.

MEYERS, T. R., S. SHORT, C. FARRINGTON, K. LIPSON, H. J. GEIGER, AND R. GATES. 1993. Establishment of negative-positive threshold optical density value for the enzyme-linked immunosorbent assay (ELISA) to detect soluble antigen of *Renibacterium salmoninarum* in Alaskan Pacific salmon. Diseases of Aquatic Organisms 16: 191–197.

MIRIAM, A., S. G. GRIFFITHS, J. E. LOVELY, AND W. H. LYNCH. 1997. PCR and Probe-PCR assays to monitor broodstock Atlantic salmon (*Salmo salar* L.) ovarian fluid and kidney tissues for the presence of DNA of the fish pathogen *Renibacterium salmoninarum*. Journal of Clinical Microbiology 35: 1322–1326.

MITCHUM, D. L., L. E. SHERMAN, AND G. T. BAXTER. 1979. Bacterial Kidney Disease in feral populations of brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), and rainbow trout (*Salmo gairdneri*). Journal of Fish Research Board Canada 36: 1370–1376.

OLEA, I., D. W. BRUNO, AND T. S. HASTINGS. 1993. Detection of *Renibacterium salmoninarum* in naturally infected Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum) using an enzyme-linked immunosorbent assay. Aquaculture 116: 99–110.

PASCHO, R. J., AND D. MULCAHY. 1987. Enzyme-linked

immunosorbent assay for a soluble antigen of *Renibacterium salmoninarum*, the causative agent of salmonid bacterial kidney disease. Canadian Journal of Fisheries and Aquatic Science 44: 183–191.

———, AND D. G. ELLIOTT. 2004. Bacterial kidney disease. *In* American Fisheries Society—fish health section blue book: Suggested procedures for the detection and identification of certain finfish and shellfish pathogens. American Fisheries Society, Bethesda, Maryland.

———, D. CHASE, AND C. L. MCKIBBEN. 1998. Comparison of the membrane-filtration fluorescent antibody test, the enzyme-linked immunosorbent assay and the polymerase chain reaction to detect *Renibacterium salmoninarum* in salmonid ovarian fluid. Journal of Veterinary Diagnostic Investigation 10: 60–66.

PATERSON, W. D., C. GALLANT, D. DESAUTELS, AND L. MARSHALL. 1979. Detection of bacterial kidney disease in wild salmonids in the Margaree River system and adjacent waters using an indirect fluorescent antibody technique. Journal of Fish Research Board Canada 36: 1464–1468.

SAKAI, M., S. ATSUTA, AND M. KOBAYASHI. 1989. Comparison of methods used to detect *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease. Journal of Aquatic Animal Health 1: 21–24.

SAMI, S., T. FISCHTER-SCHERL, R. W. HOFFMAN, AND C. PFEIL-PUTZIEN. 1992. Immune complex mediated glomerulonephritis associated with bacterial kidney disease in the rainbow trout (*Oncorhynchus mykiss*). Veterinary Pathology 29: 169–174.

SANDERS, J. E., AND J. L. FRYER. 1980. *Renibacterium salmoninarum* gen. nov., sp. nov., the causative agent of bacterial kidney disease in salmonid fishes. International Journal of Systematic Bacteriology 30: 496–502.

SNIESZKO, S. F., AND P. J. GRIFFIN. 1955. Kidney disease in brook trout and its treatment. Progressive Fish Culturist 17: 3–13.

TURAGA, P. S., G. D. WEINS, AND S. L. KAATTARI. 1987. Analysis of *Renibacterium salmoninarum* antigen production in situ. Fish Pathology 22: 209–214.

WHITE, M. R., C. C. WU, AND S. R. ALBREGTS. 1995. Comparison of diagnostic tests for bacterial kidney disease in juvenile steelhead trout (*Oncorhynchus mykiss*). Journal of Veterinary Diagnostic Investigation 7: 494–499.

WIENS, G. D., AND S. L. KAATTARI. 1991. Monoclonal antibody characterization of a leukoagglutinin produced by *Renibacterium salmoninarum*. Infection and Immunity 59: 631–637.

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