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Recovery of *Renibacterium salmoninarum* from naturally infected salmonine stocks in Michigan using a modified culture protocol

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KEYWORDS

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Abstract *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD), is a fastidious and slow-growing bacterium that is extremely difficult to grow *in vitro*. Herein, we describe a modified primary culture protocol that encompasses a modified bacteriological culture medium and a tissue processing procedure. In order to facilitate the release of *R. salmoninarum* from granulomatous tissues, kidneys of infected fish were homogenized in a high speed stomacher. The kidney disease medium (KDM2), routinely used for primary culture of *R. salmoninarum* was modified by the addition of antibiotics and metabolites. When a relatively large inoculum of diluted kidney homogenate was streak-plate inoculated onto the modified KDM2, colonial growth of *R. salmoninarum* was achieved within 5–7 days, compared to the standard of two weeks or more. The modified procedure was then used to determine the prevalence of *R. salmoninarum* among representative captive and feral salmonid stocks in Michigan. Prevalence and clinical manifestations varied among species, strains of fish, and locations; however, *R. salmoninarum* isolates were biochemically homogenous. The improved primary culture procedure described in this study enabled

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selective and quick isolation of *R. salmoninarum*. Also, the isolates retrieved in this study constitute a unique biological resource for future studies of *R. salmoninarum* in the Laurentian Great Lakes.

Introduction

Bacterial kidney disease (BKD), caused by the gram positive bacterium *Renibacterium salmoninarum*, is a serious disease threatening salmonids all over the northern hemisphere [1]. *R. salmoninarum* elicits the formation of granulomatous tissues, primarily in hematopoietic tissues of the kidneys, thereby impairing the vital functions of this important organ [2]. The bacterium is transmitted both horizontally and vertically, through gamete to offspring, a matter that poses a great challenge for BKD control. In the Laurentian Great Lakes, BKD was first described in the early 1950s [3] and has since spread and became endemic in the entire basin [4,5]. In the late 1980s, BKD prevalence reached untold levels in wild and hatchery-propagated salmonids, and was associated with widespread mortality of wild salmonids in Lake Michigan [6,7]. In the absence of an effective vaccine, BKD control relies primarily on culling infected wild or captive spawning adults and resulting egg lots, and treating the broodstock fish or fertilized eggs with a drug such as erythromycin. Regulatory agencies in States and Canadian provinces bordering the Great Lakes enacted continuous monitoring and surveillance of susceptible fish species in order to determine the extent of this disease in the different water bodies [4,5].

Obtaining accurate prevalence data of *R. salmoninarum* infections in carrier, apparently healthy fish is difficult due to the inconsistent successes to isolate the bacterium in culture. *R. salmoninarum* is fastidious in it's requirement for *l*-cysteine and is very slow growing, a matter that allows the overgrowth of bacterial and fungal contaminants upon primary isolation. Moreover, the layers of granulomatous tissues that the host forms around infection foci make the bacterium difficult to attain for isolation using standard bacterial recovery methods. This problem is further complicated by the uneven distribution of *R. salmoninarum* aggregations within affected kidney tissues, especially in asymptomatic fish [8,9], and the presence of inflammatory mediators that inhibit *R. salmoninarum in vitro* growth [10,11]. The culture medium routinely used for the isolation of *R. salmoninarum* is the kidney disease medium (KDM2) developed by Evelyn [12], which allows bacterial growth within 12 weeks. Later, Evelyn et al. [13,14] noted that when filter-sterilized broth that had previously been used to grow *R. salmoninarum* (spent medium) was used to supplement fresh KDM2, *R. salmoninarum* colonial growth was improved and the incubation time to visualize colonies was reduced. The improved growth was attributed to metabolites secreted by the initial culture. Using the same concept, Teska [15] and Starliper et al. [16] incorporated *R. salmoninarum* spent medium (1% v/v) into KDM2 for related BKD studies. While these modifications have supported the subculturing of previously isolated *R. salmoninarum* strains, direct isolation from infected tissues, particularly from carrier fish, yielded inconsistent results.

Despite the widespread distribution of BKD in the Great Lakes, only a few number of *R. salmoninarum* isolates were retrieved from resident fish [17,18]. Among the retrieved isolates, those from the Michigan side of Lake Michigan were the most genetically diverse [18] and were of higher virulence [19] when compared to isolates obtained from other locations in the USA and the world. To better understand the biological and genetic diversity of Great Lakes *R. salmoninarum*, there is a need to retrieve a greater number of isolates. To this end, we present a combination of a modified tissue processing protocol and culture medium to enhance the primary isolation of *R. salmoninarum*. The modified protocol was then used to determine *R. salmoninarum* prevalence in representative captive and feral salmonine stocks in Michigan.

Material and methods

Modification of the kidney disease medium (KDM2)

Standard KDM2 was modified by supplementing its components with 10% fetal calf serum, four antimicrobials, and filtered (0.45 μM) 1% *R. salmoninarum* spent medium (metabolite). These modifications combined the observations that each of these supplements enhance and/or select *R. salmoninarum* growth [14–16,20]. The modified medium will be referred to as the modified KDM2 (MKDM). Briefly, MKDM consists of peptone (1% w/v), yeast extract (0.05% w/v), *l*-cysteine HCl (0.1% w/v), cycloheximide (0.005% w/v), new born calf serum (10% v/v), filter-sterilized *R. salmoninarum* spent broth (1% v/v), oxolinic acid (0.00025% w/v), polymyxin B sulfate (0.0025% w/v), D-cycloserine (0.00125% w/v), and agar (1.5% w/v). The pH was adjusted to 6.8 using 1 N NaOH. 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).

Modified protocol of fish tissue processing and plating to enhance R. salmoninarum recovery

Kidney tissues were collected from 515 wild adult salmon returning to spawn in Michigan's gamete collecting stations (weirs). Fish included 150 returning chinook salmon (*Oncorhynchus tshawytscha*) collected from the Little Manistee River Weir (LMRW) at Manistee, Michigan (Lake Michigan watershed), the Swan River Weir (SRW) at Rogers City, Michigan (Lake Huron watershed) and the Platte River Weir (PRW) at Beulah, Michigan (Lake Michigan watershed). An additional 165 Michigan-adapted coho salmon (*Oncorhynchus kisutch*) strain and 56 Hinchenbrook coho salmon strain were collected from the Platte River Weir (PRW). Kidney tissues were also collected from captive broodstock collected from Michigan state fish hatcheries including 60 brook trout (*Salvelinus fontinalis*) of the Iron River strain, 60 lake trout (*Salvelinus namaycush*) that were kept in raceways receiving surface water from the Cherry Creek (Lake Superior watershed) at the Marquette State Fish Hatchery (MSFH), Marquette, MI. Additionally, kidneys were collected from 12 brown trout (*Salmo trutta*) of the Wild Rose strain and 12 rainbow trout

(*Oncorhynchus mykiss*) of the Eagle Lake strain were collected from Oden State Fish Hatchery (OSFH) at Alanson, MI (Lake Michigan watershed). Males and females were equally represented from each sample origin.

Additional kidney tissue samples were obtained from 495 hatchery-reared fingerlings collected in the spring of 2003 from state fish hatcheries including: 60 brook trout (Assinica strain), 60 brook trout (Iron River strain) from MSFH, 120 lake trout from MSFH, 60 brown trout (Wild Rose strain), 60 brown trout (Seeforellen strain), 60 brown trout (Gilchrist strain) from OSFH, and 60 rainbow trout (Eagle Lake strain) from OSFH. Additionally, in August 2004, 15 brook trout (unknown strain) fingerlings were sampled from a private aquaculture facility at Harrisonville, MI.

Fish were sacrificed by immersion in carbon dioxide-laden water or with an overdose of MS222 (Finquel, Argent Chemical Laboratories, Redmond, WA). The abdominal cavity was cut open to examine internal organs for clinical signs associated with BKD, followed by the collection of kidney tissue samples. In all fish, the entire kidneys (from the skull to the end of the peritoneal cavity) were collected. Kidney tissues were either processed immediately or stored individually at −80 °C until processed. Cross contamination of samples was avoided by sterilizing dissecting tools following the necropsy of each fish.

Attempts to isolate *R. salmoninarum* from kidney tissues were performed by each of the following procedures: (a) streak-plate inoculating a 10 μl loopful of kidney tissues onto MKDM plates, (b) 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 streak-plate inoculating 100 μl inoculum of the suspension onto MKDM, or (c) placing kidney tissues in 7.5 × 18.5 cm Whirl-Pak® bags (Nasco, Fort Atkinson, WI, USA), suspending in HBSS (1:4 w/v), then crushing the suspension in a Biomaster Stomacher-80 (Wolf Laboratories Limited, Packlington, York, UK) at the highest speed setting for 120 s. One hundred microliters of the suspension was added to one end of an MKDM plate and then spread over the surface using a sterile bacteriological loop. All 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 suspect R. salmoninarum isolates

All suspect *R. salmoninarum* colonies having characteristic colony morphology were subcultured for confirmation [1,21]. The following biochemical tests were performed for each isolate: Gram staining, 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), arginine dihydrolase, urease, hydrolysis of Tween 20, 40, 60, 80, production of indole, methyl red, DNAse test using DNAse test medium (Remel). Carbohydrate utilization was performed using a basal media (DIFCO-BD) supplemented with each filter-sterilized (0.45 μM) sugar to obtain a final concentration of 1%; one exception was salicin, which was made to a 0.5% final concentration. The following sugars (Sigma) were evaluated: arabinose, glucose, lactose, maltose, rhamnose, salicin, sucrose, sorbitol, and xylose. Results of biochemical tests were matched against standard *R. salmoninarum* biochemical characters described elsewhere [22].

Nested polymerase chain reaction

Suspect *R. salmoninarum* bacterial colonies were also identified using primers which amplify a region of the gene encoding the *R. salmoninarum* p57 antigen in a nested polymerase chain reaction (nPCR) as described elsewhere [23]. Bacterial DNA was extracted using the DNeasy Extraction Tissue Kit (Qiagen Inc., Valencia, CA). Pelleted bacteria were lysed with a solution that consisted of lysozyme (Sigma), *Tris*-HCl, EDTA (Sigma) and Triton X100 (Sigma) at 37 °C for 1 h. The *n*PCR protocol followed that recommended by the American Fishery Society, Fish Health Section [24]. Detection of an amplicon of 320 bp confirmed the *R. salmoninarum* identity of suspected colonies. Confirmed *R. salmoninarum* isolates were cryopreserved and deposited at the Aquatic Animal Health Laboratory, Michigan State University, East Lansing, MI. Bacterial suspensions were prepared from 5 day-old cultures in MKDM broth (not supplemented with antibiotics) and then stored at −80 °C.

Susceptibilities to antibiotics

Two media were used to test the isolates for sensitivity to antibiotics using a modified Kirby–Bauer disc diffusion method [25]: (a) Antimicrobial-free MKDM agar medium, (b) Modified Mueller Hinton agar medium (MMHA), which was supplemented with 0.01% *l*-cysteine HCl (Sigma), 0.05% yeast extract (Sigma), and 10% fetal bovine serum (Sigma). Antibiograms were developed for 12 representative *R. salmoninarum* isolates. Five-day-old colonies were suspended in sterile saline (0.85% NaCl) to obtain turbidity equivalent to a 0.5 McFarland standards (Remel). From each bacterial suspension, 200 μl volumes were spread onto antibiotic-free MKDM and MMHA plates to create uniform lawns of bacterial growth. Using an automatic dispenser (Remel), antibiotic discs (5 mm in diameter) were placed on the culture plate surface. The plates were inverted and incubated at 15 °C in a subambient temperature incubator (Fisher Scientific Company L.L.C. Hanover Park, IL, USA) for 5 days. Results were recorded by measuring the diameter of the zones of inhibition in millimeters around each disc using a calibrated ruler. The following antibiotic discs (all from Remel) were used in the antibiogram: chloramphenicol, Terramycin, sulfamethoxazole–trimethoprim, carbenicillin, erythromycin, azithromycin, kanamycin, clindamycin, polymyxin B sulfate, novobiocin, ofloxacin, ciprofloxacin, enrofloxacin, and norfloxacin.

Prevalence of R. salmoninarum in Michigan salmonid stocks

Presence of *R. salmoninarum* with or without kidney nodules was recorded. Stocks of each category (wild adult salmon, captive adult trout, and captive trout fingerlings) were compared to each other. Statistical analysis was performed using the Pearson's chi square analysis using a two way contingency table unless otherwise indicated. The level for significance was designated as $P < 0.05$.

Results

Evaluation of MKDM and the modified tissue processing protocol for recovery of R. salmoninarum

When MKDM was streaked with 10 µl of infected kidney tissue, a few colonies were evident 20 days post incubation. Using minced kidney of the same tissue and increasing the inoculum to 100 µl shortened the incubation time to 15 days. However, when kidney tissue samples were homogenized in the stomacher and MKDM was inoculated with 100 µl of the stomached homogenate, profuse growth was achieved within a relatively short period (5–10 days). *R. 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 bacterial contaminants, inoculating MKDM plates with 100 µl of tissue homogenates became the routine protocol in this study for the surveillance of *R. salmoninarum* in salmonid fish stocks.

Isolation and identification of R. salmoninarum from Michigan salmonid stocks

A total of 559 *R. salmoninarum* isolates were retrieved from infected fish tissues over the two-year period of the study. All colonies were creamy-whitish, glistening, 1–2 mm in diameter, rounded, and smooth. When plates were incubated for prolonged times (40 days or more), colonies were granular white or crystalline in appearance. Gram staining demonstrated Gram-positive diplo- or coccobacilli. No capsules, metachromatic granules, or bipolarity were detected. Nested PCR performed on all isolates demonstrated the *R. salmoninarum* characteristic 320 bp band. Biochemical testing of 12 representative isolates demonstrated that they were biochemically uniform. Isolates were non-motile, and did not produce cytochrome oxidase, DNase, arginine dihydrolase, amylase, or urease, and were unable to hydrolyze esculin or Tween 80. Additionally, the isolates were unable to utilize the assayed carbohydrates, did not produce indole, and yielded negative methyl red reactions. However, all isolates were catalase positive and able to hydrolyze Tween 20, 40, and 60. The biochemical results conform perfectly to those described previously [26].

The antibiograms indicated that after 5–10 days incubation, the inhibition zones obtained from isolates cultured on antimicrobial-free MKDM medium were sharper and more obvious than those obtained by culture on MMHA medium. However, both media yielded similar results. All 12 isolates were highly sensitive to enrofloxacin and ciprofloxacin. The isolates were markedly sensitive to ofloxacin, norfloxacin, sulfamethoxazole–trimethoprim, Terramycin, chloramphenicol, novobiocin, and carbenicillin. All isolates were resistant to polymyxin B and clindamycin, and kanamycin. 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 both macrolide antibiotics.

Prevalence of R. salmoninarum and BKD clinical signs in Michigan's salmonid stocks

The findings demonstrate that *R. salmoninarum* continues to be prevalent in wild chinook and coho salmon stocks (Table 1). A total number of 305 confirmed *R. salmoninarum* isolates were retrieved from 371 chinook and coho salmon (82.2%). Comparisons among the five wild salmon populations using the Pearson Chi-Square Test revealed the presence of marked significant differences in prevalence ($\chi^2 = 39.2999$, $df = 4$, P-value $= 6.04 \times 10^{-8}$) with coho salmon (Hinchenbrook strain) being the highest and chinook salmon being the lowest. In general, *R. salmoninarum* prevalence from coho salmon (the two stocks combined) was higher compared to the three chinook salmon groups combined ($\chi^2 = 14.4916$, $df = 1$, P-value $= 0.0001408$). However, there were no significant

Table 1 Isolation of *Renibacterium salmoninarum* from chinook and coho salmon returning spawners. Samples were collected in the fall of 2002 from the Little Manistee River Weir (LMRW, Lake Michigan watershed), the Platte River Weir (PRW, Lake Michigan watershed), and the Swan River Weir (SRW, Lake Huron watershed).

Host	Weir	Number of fish tested	Prevalence (*R. salmoninarum* positive/total)		
			Apparently healthy fish	Fish with kidney lesions characteristic of BKD	Total
Chinook salmon	LMRW	63	47/63 (74.60%)	5/63 (7.93%)	52/63 (82.53%)
	SRW	60	26/60 (43.33%)	3/60 (5%)	29/60 (48.33%)
	PRW	27	23/27 (85.18%)	3/27 (11.11%)	26/27 (96.29%)
Coho salmon (MI–adapted strain)	PRW	165	112/165 67.87%)	30/165 (18.18%)	142/165 (86%)
Coho salmon (Hinchenbrook strain)	PRW	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 2 Prevalence of *Renibacterium salmoninarum* in captive trout broodstocks. Samples were collected in the fall 2002 from Michigan state fish hatcheries.

Species	Number of fish examined	Fish from which confirmed *R. salmoninarum* culture was obtained/total		
		Apparently healthy	Fish with kidney lesions characteristic of BKD	Total
Brook trout (Iron River strain)	60	22/60 (36.66%)	10/60 (16.66%)	32/60 (53.33%)
Lake trout	60	40/60 (66.66%)	3/60 (5%)	43/60 (71.66%)
Brown trout (Wild Rose strain)	12	8/12 (66.66%)	4/12 (33.33%)	12/12 (100%)
Rainbow trout (Eagle Lake strain)	12	9/12 (75%)	2/12 (16.66%)	11/12 (91.66%)
Total	144	79/114 (54.9%)	19/144 (13.2%)	98/144 (68%)

differences in the prevalence ($\chi^2 = 0.0697$, d$f = 2$, P-value = 0.9658) among the three chinook salmon stocks. On the other hand, there were significant differences in prevalence ($\chi^2 = 18.5102$, d$f = 1$, P-value = 1.69×10^{-5}) between the Michigan-adapted and coho salmon. Less than 20% of the fish (71 out of 371) examined exhibited the typical signs of BKD with coho salmon (Hinchenbrook strain) being the highest and SRW-chinook salmon the lowest ($\chi^2 = 58.7887$, d$f = 4$, p-value = $5\ .212 \times 10^{-12}$). In addition to the increased prevalence of *R. salmoninarum*, the number of coho salmon with BKD signs was significantly higher than those in the three chinook salmon populations ($\chi^2 = 33.8169$, d$f = 1$, p-value = 6.055e-09). There were no significant differences in clinical signs of BKD among the three chinook salmon stocks ($\chi^2 = 0.7273$, d$f = 2$, P-value = 0.6951), or the two coho salmon stocks.

Results also demonstrated that clinical BKD is also present in captive broodstocks, albeit at a lower rate (13.2%) than in the two salmon stocks with brown trout being the highest (33.33%). Brown trout (Wild Rose) also exhibited the highest *R. salmoninarum* prevalence also, while lake trout was the lowest ($\chi^2 = 8.1579$, d$f = 3$, P-value = 0.04286). Overall, *R. salmoninarum* was isolated and confirmed from 98 fish out of 144 fish tested (68%). Comparisons among the four populations using the Pearson Chi-Square Test revealed the presence of significant differences in prevalence ($\chi^2 = 8.6225$, d$f = 3$,

Table 3 Prevalence of *Renibacterium salmoninarum* in hatchery-raised fingerlings. All samples were collected from two state fish hatcheries in January 2003, while farmed brook trout was obtained from a commercial aquaculture facility in August 2004.

Fish species (strain)	Number of fish tested	Fish from which confirmed *R. salmoninarum* culture was obtained/total		
		Apparently healthy	Fish with kidney lesions characteristic of BKD	Total
Brook trout (Assinica)	60	15/60 (25%)	10/60 (16.7%)	25/60 (41.7%)
Brook trout (Iron River)	60	20/60 (33.3%)	30/60 (50%)	50/60 (83.3%)
Farmed brook trout	5	0/15 (0%)	9/15 (60%)	9/15 (60%)
Lake trout	120	22/120 (18.3%)	0/120 (0%)	22/120 (18.3%)
Brown trout (Wild Rose)	60	8/60 (13.3%)	2/60 (3.3%)	10/60 (16.7%)
Brown trout (Seeforellen)	60	14/60 (23.3%)	1/60 (1.7%)	15/60 (25%)
Brown trout (Gilchrist)	60	12/60 (20%)	3/60 (5%)	15/60 (25%)
Rainbow trout (Eagle Lake)	60	8/60 (13.3%)	2/60 (3.3%)	10/60 (16.7%)
Total	495	99/495 (20%)	57/495 (11.5%)	156/495 (31.5%)

P-value = 0.03476 and *P*-value = 0.01919 by Fisher's Exact Test) with brown trout (Wild Rose strain) being the highest and brook trout (Iron River strain) being the lowest.

Prevalence of R. salmoninarum and BKD clinical signs in propagated trout fingerlings

R. salmoninarum was also prevalent in hatchery-propagated fingerlings. From hatchery fish, a total of 156 confirmed isolates were obtained from the 495 fish examined (31.5%). Differences in prevalence among fish species and strains within the species were evident ($\chi^2 = 50.1976$, d$f = 7$, *P*-value = 1.321×10^{-8}). Brook trout (Iron River) had the highest prevalence, whereas the Brown trout (Wild Rose) and Rainbow trout were lower. There were also significant differences in prevalence ($\chi^2 = 9.9429$, d$f = 2$, *P*-value = 0.006933 and *p*-value = 0.004097 by Fisher's Exact Test) among the three brook trout strains. On the other hand, there were no significant differences in prevalence ($\chi^2 = 1.3072$, d$f = 2$, *P*-value = 0.5202 and *p*-value = 0.6218 by Fisher's Exact Test) among the three Brown trout strains. Fish with BKD renal nodules constituted 11.5% of the fish examined (57 out of 495) with the farmed brook trout being the highest and lake trout is the lowest ($\chi^2 = 97.2456$, d$f = 7$, *P*-value < 2.2×10^{-16}). Again, there were significant differences among the three brook trout strains ($\chi^2 = 17.1837$, d$f = 2$, *P*-value = 0.0001856) in exhibiting BKD clinical signs, while no significant differences were noticed among the three brown trout strains ($\chi^2 = 1$, d$f = 2$, *P*-value = 0.6065) (Table 3).

Discussion

Data from this study demonstrated that the combined use of MKDM medium and stomacher-homogenized tissues diluted in HBSS was very effective for primary isolation of *R. salmoninarum* from large sample sizes (i.e., number) of fish. The combination of antimicrobial supplementation and the relatively short incubation period minimized the growth of contaminating bacteria and fungi. There are a number of factors that might have resulted in the improved growth of *R. salmoninarum* that we noted. First, the homogenization of kidney tissues affected the release of the intracellular *R. salmoninarum* from the granulomas and fibrous tissue layers. Second, the relatively aggressive tissue processing may have led to the release of *R. salmoninarum* metabolites that facilitated quicker *in vitro* bacterial growth and perhaps, increased the percent of metabolite supplement above 1%. Third, the entire kidney tissues (posterior and anterior) were used for the isolation, a procedure that increases the likelihood of isolating bacteria even if present in low numbers, such as in the case of carrier fish. Pascho et al. [27] were able to double the likelihood of isolating *R. salmoninarum* from infected fish by combining samples taken from three different sites in the kidneys of individually tested fish. Fourth, the use of a large inoculum volume (100 µl) enhanced the likelihood of bacterial isolation when compared to using lesser volumes. Fifth, mixing kidney tissues with four times their weight of HBSS may have diluted inhibitory molecules present in tissue [10,11,28] that are thought to reduce the likelihood of isolating *R. salmoninarum* from homogenized kidney tissues. Finally, the unique formula of HBSS with its rich inorganic ions and electrolyte content might have contributed important growth factors and buffered 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.

Our results from conventional biochemical testing revealed that Michigan-origin *R. salmoninarum* isolates coincided with the standard biochemical criteria previously reported [1,22,26,29]. The biochemical results also indicated uniformity among Michigan isolates. Our identifications were further confirmed by nPCR results as all *R. salmoninarum* isolates exhibited the 320 bp band characteristic for *R. salmoninarum* [23].

The antibiogram performed on 12 *R. salmoninarum* representative isolates showed that results for Michigan-origin isolates also were similar to previous reports [30–33]. However, our results of isolate resistances to erythromycin and azithromycin (Oden BNT-BS-02 and Oden BNT-BS1-02), were unexpected since the "wild-type" *R. salmoninarum* strains are known to be sensitive to macrolide antibiotics [30]. These results corroborate with the results of Rhodes et al. [34] who recently reported the emergence of *R. salmoninarum* strains with decreased sensitivity to erythromycin.

From the survey performed in this study, a number of interesting results were demonstrated that would vastly improve our current understandings of the status of endemic *R. salmoninarum* and BKD in Michigan, as well as help shape the design of future epidemiological studies. It is quite clear that *R. salmoninarum* is widespread among Michigan's wild and hatchery-reared fish populations, as it has been isolated from every fish population and strain that was tested (Tables 1–3). Infected fish are apparently not only carriers, but some fish develop clinical kidney lesions consistent with BKD. Vertical transmission seems to play an important role in *R. salmoninarum* transmission among brown and rainbow trout because fingerlings were kept throughout their life in raceways supplied with pathogen-free well water and have presumably not been exposed to an external source of *R. salmoninarum*. Our results also suggested that *R. salmoninarum* prevalence varies among the host species examined, with brook trout and coho salmon being the highest in both prevalence and presence of kidney lesions. Furthermore, even within the same host species, fish strain differences seem to play a role in relative susceptibility to *R. salmoninarum*. For example, the Hinchenbrook coho salmon strain showed a higher prevalence than the Michigan-adapted coho salmon strain collected from Platte River. Also, the Hinchenbrook coho salmon strain showed a relatively higher percentage 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) and is perhaps more naïve, while the other coho salmon strain has adapted to the Great Lakes basin since its introduction in the 1960s (Edward Eisch, Michigan Department of Natural Resources, personal communication). Relative susceptibilities also apply to the brook trout, as evidenced by the Iron River strain, which has recently been adopted for propagation purposes. In contrast, 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. [35] who made a similar observation with steelhead trout (*O. mykiss*). Lastly, in the case of chinook salmon, results suggested 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 percent of clinical cases (11%) when compared to Swan River chinook salmon (48% and 5%, respectively) that came from Lake Huron. The same trend was observed in chinook salmon from the Little Manistee Weir, which exhibited a higher *R. salmoninarum* prevalence than chinook salmon from the Swan River weir. Spatial distribution and population density could play a role in elevating the prevalence of BKD among fish populations [36].

Conclusions

The modifications made to the culture medium and tissue processing methods proved effective in facilitating the primary isolation of *R. salmoninarum* from fish tissues. We highly recommend this modified procedure for use in future epizootiological studies. The 276 *R. salmoninarum* isolates retrieved in this study constitute an important resource for further *R. salmoninarum* and BKD studies worldwide.

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