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FIRST RECORD OF *RENIBACTERIUM SALMONINARUM* IN THE SEA LAMPREY (*PETROMYZON MARINUS*)

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ABSTRACT: Bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum*, is a widespread problem with major implications for salmonid fish species. The mechanisms by which the bacterium has 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 because of the ecologic complexity of heterogeneous habitats and the lack of adequate funding. Herein, we report on the isolation of *R. salmoninarum* from the kidneys of the sea lamprey (*Petromyzon marinus*). The bacterium was cultured from kidneys of 16% and 4% of lampreys collected from two locations within the Lake Ontario watershed in 2003 and 2004, respectively. The identity of bacterial colonies was verified with the nested polymerase chain reaction and quantitative enzyme-linked immunosorbent assay.

Key words: Bacterial Kidney Disease, Great Lakes, *Petromyzon marinus*, *Renibacterium salmoninarum*, sea lamprey.

INTRODUCTION

Bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum*, is a serious bacterial disease of salmonines (Fryer and Sanders, 1981) that is widespread in the Great Lakes basin (Faisal and Hnath, 2005). Although the epidemiology of this disease is not completely understood, most studies suggest that *R. salmoninarum* infects salmonines exclusively and that carrier fish are responsible for its distribution and transgenerational transmission (Wood and Yasutake, 1956; Bullock and Herman, 1988). Results from other studies, however, have indicated that nonsalmonine fish species such as the Pacific hake (*Merluccius productus*, Kent et al., 1998) and the Pacific herring (*Clupea harengus pallasi*, Paclibare et al., 1988) may harbor *R. salmoninarum*, but the potential role of nonsalmonid fish species in the epidemiology of BKD remains to be elucidated fully.

In the Great Lakes basin, a number of nonindigenous invasive species have caused serious ecologic and economic losses; among these is the jawless sea lamprey (*Petromyzon marinus*). 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. Despite the use of physical and electrical sea lamprey barriers beginning in the mid-1940s, and the advent of chemical control in 1958 (Smith and Tibbles, 1980), these two fisheries have not fully recovered.

To reduce the numbers of sea lampreys further and limit their distribution, the Great Lakes Fishery Commission (GLFC), mandated by a binational convention to coordinate sea lamprey control in the Great Lakes basin, actively supports a policy of integrated management. In addition to lampricide application, the GLFC relies on alternative technologies, such as the chemical sterilization of males. This began in the early 1990s and is based on trapping male sea lampreys, sterilizing them, and releasing sterile males into streams to compete with fertile males for spawning females (Twohey et al., 2003).

Field assessments indicate a decreased sea lamprey hatch rate in streams where the sterile-male-release technique is utilized (Bergstedt et al., 2003). Currently up to 40,000 males are collected annually from different source streams throughout the Great Lakes basin, transported to a sterilizing facility in Hammond Bay, Michigan, then all are transported for release into the St. Mary's River (the connecting channel between lakes Superior and Huron). These transfers of lampreys from and to different locations in the Great Lakes basin may concurrently transfer various pathogens, and this probability has raised concerns related to resident fish health. In response to these concerns, GLFC initiated a screening program to determine if sea lampreys might be contributing to within-basin spread of fish diseases. In the course of these screenings, the sea lamprey was identified, for the first time, as a new host for *R. salmoninarum*.

MATERIALS AND METHODS

In the early summer of 2003, 25 adult spawning-phase sea lampreys were moved concomitantly from two Lake Ontario streams, the Humber River (43°39′16′′N, 79°29′45′′W) and Duffins Creek (43°50′54′′N, 79°03′25′′W), and presented alive to the Aquatic Animal Health Laboratory (AAHL) at Michigan State University. This stream and river represented potential source sites for providing sterilized males to the St. Mary's River, which was the target stream. In early summer 2004, an additional 118 adult sea lampreys were caught from Duffins Creek (n=58) and the Humber River (n=60). These were held separately and transported alive to the AAHL. Lampreys were kept in well-aerated, chilled (10 C) water tanks until examined.

Lampreys were euthanized using an overdose of MS 222 (tricaine methane sulfonate, Argent Chemical Laboratories, Redmond, Washington, USA) and dissected under aseptic conditions. Kidneys were removed aseptically and placed in sterile 7.5 cm × 18.5 cm Whirl-Pak® bags (Nasco, Fort Atkinson, Wisconsin, USA). Hank's Balanced Salt Solution was then added to the Whirl-Pak® at a ratio of 1:4 (weight/volume), and kidney was homogenized for 120 sec using a high-speed Biomaster Stomacher-80 (Wolf Laboratories Limited, Pocklington, York, UK).

Homogenized kidney tissue aliquots (100 µl) were spread onto modified kidney diseases medium (MKDM; Eissa, 2005), and inoculated plates were incubated for up to 20 days in a subambient temperature incubator adjusted to 15 C. Culture plates were checked every day for colonial growth. The morphological description of isolates was based on both colony morphology and Gram stain. Identification of the isolates was performed according to the standard morphological criteria for *R. salmoninarum* (Sanders and Fryer,1980; Austin and Austin,1999) and a number of conventional biochemical tests. These included motility, using motility test medium (BD Diagnostics, Sparks, Maryland, USA), cytochrome oxidase with Pathotec strips (Remel, Lenexa, Kansas, USA), catalase test with 3% hydrogen peroxide, bile esculin using bile esculin agar (Remel), and DNAse test using DNAse test medium (Remel). Results of the biochemical tests were compared against standard *R. salmoninarum* biochemical characters described by Bruno and Munro (1986).

Molecular confirmation of the isolates was conducted using a nested polymerase chain reaction (nPCR) according to the method described by Chase and Pascho (1998). A DNeasy tissue extraction kit (Qiagen, Valencia, California, USA) was used for the extraction of DNA from 100 µl 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 min at 4 C, and the pellets were incubated with lysozyme buffer consisting of 180 µl of 20 mg lysozyme (Sigma Chemical, St. Louis, Missouri, USA), 20 mM Tris-HCl, pH 8.0, 2 mM EDTA (Sigma), and 1.2% (v/v) Triton X 100 (Sigma) at 37 C for 1 hr. 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 PCR and 2 µl for second PCR), water, and master mixes (45 µl for first PCR and 48 µl for second PCR). Controls were composed of a PCR mixture containing no DNA template reagent (negative control), positive *R. salmoninarum*, and positive tissue control. For electrophoresis, 10 µl of nPCR product and controls were mixed with 2 µl of 6X loading dye (Sigma) and loaded on a 2% agarose gel (Invitrogen Life Technologies, Carlsbad, CA). Each gel included a 1 kbp DNA ladder with 100 bp increments (Invitrogen). Gels were run

TABLE 1. Prevalence of *Renibacterium salmoninarum* in kidneys and blood of 2003–2004 Lake Ontario sea lampreys using nested polymerase chain reaction (nPCR) and quantitative enzyme-linked immunosorbent assay (Q-ELISA) and culture.

Year and sample nos.	Site of collection	Type of sample	% positive cultured samples	% positive nPCR samples	% positive Q-ELISA samples
2003 SL1-25-03	Humber/Duffins	Kidney	(4/25) 16%	(4/25) 16%	ND
2003 SL1-25-03	Humber/Duffins	Blood	(0/25) 0%	ND	ND
2004 SLHR1-60	Humber River	Kidney	(4/60) 6.7%	(3/60) 5%	(2/60) 3%
2004 SLHR1-60	Humber River	Blood	(0/60) 0%	ND	ND
2004 SLDC1-58	Duffins Creek	Kidney	(1/58) 2%	(38/58) 66%	0%
2004 SLDC1-58	Duffins Creek	Blood	(0/58) 0%	ND	ND

ND = Not Done.

in 1 X Tris acetate gel buffer (Sigma). Gels were visualized under the KODAK EDAS Camera System and UV Trans-illuminator. Samples were considered positive when a 320 base pair (bp) band was detected.

Sample preparation and quantitative enzyme-linked immunosorbent assay (Q-ELISA) protocols were adopted from the methods detailed in Pascho and Mulcahy (1987) and Pascho et al. (1998). The positive-negative threshold was determined according to the calculations detailed in Meyers et al. (1993). Absorbance above 0.10 was considered positive. 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

Renibacterium salmoninarum was isolated from the kidneys of four of 25 (2003) and five of 118 (2004) adult sea lampreys from the Duffins Creek/Humber River assemblage. The organism was not detected in blood samples tested in 2003 and 2004 (Table 1). 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 motility test negative, catalase test positive, cytochrome oxidase test negative, bile esculin degradation negative, DNAse test negative, and carbohydrate fermentation negative (Table 2). All the retrieved *R. salmoninarum* isolates were confirmed using nPCR and Q-ELISA.

Using nPCR, *R. salmoninarum* was detected in four (16%) of 25 lamprey kidney samples collected in the early summer of 2003 (Table 1). Using the same technique, the organism was detected in 38 (66%) of 58 lamprey kidney samples collected in 2004 from the Duffins Creek site (Table 1), and in three (5%) of 60 lamprey kidney samples collected from the Humber River site that same year. When the Q-ELISA technique was performed on the 2004 samples, *R. salmoninarum* antigens were detected in the kidneys of two of the 118 lampreys (1.7%); antigen load was classified as low (Table 1).

DISCUSSION

This is the first report of *R. salmoninarum* from sea lampreys. Although described as a salmonid-specific pathogen, *R. salmoninarum* was isolated from Lake Ontario sea lampreys in two successive years (2003 and 2004). The prevalence in sea lampreys is relatively low when compared to the prevalence found in salmonines. Despite the isolation of *R. salmoninarum* from the kidneys of sea lampreys, the bacterium was not isolated from the blood nor any other internal organs. It appears that sea lamprey *R.*

TABLE 2. Confirmation of *Renibacterium salmoninarum* isolates using culture-based assays and serologic and molecular assays.

Isolate ID	Culture-based assays[a]						nPCR[a]	Q-ELISA[a]
	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	+	—	—	—	—	—	+	+

Isolate ID: SL3, SL21, SL11, and SL14 were the isolates retrieved from adult sea lampreys in midsummer 2003. DC6 was an isolate retrieved from adult sea lamprey no. 6 collected from Duffins Creek. HR9, HR14, HR15, and HR16 were isolates retrieved from adult sea lampreys collected from the Humber River. Both DC and HR isolates were retrieved from adult sea lampreys collected in midsummer 2004.

Abbreviations of assays: C = Catalase test; O = Oxidase test; E =Esculin hydrolysis; D = DNAse test; CHO = Carbohydrate utilization; M = Motility test; nPCR = Nested PCR; Q-ELISA = Polyclonal-antibody-based quantitative ELISA.

salmoninarum isolates possess the same affinity for kidney tissues as that of salmonid isolates.

The morphologic criteria and biochemical reactions of the 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).

Although 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 consistent with these results. 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. Conversely, in the case of Duffins Creek samples, nPCR consistently yielded positive results while *R. salmoninarum* was isolated from a single lamprey (SLDC6). 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 to be 100-colony forming units/gram tissue in salmonid fish (Lee, 1989). The nested PCR assay can detect as few as four to 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 lampreys in the epidemiology of BKD in the Great Lakes requires further investigation.

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