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FIRST RECORD OF *FLAVOBACTERIUM PSYCHROPHILUM* INFECTION IN THE SEA LAMPREY (*PETROMYZON MARINUS*) FROM LAKE ONTARIO

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SUMMARY

During health inspection of a group of sea lampreys, collected from Lake Ontario, for their suitability to relocation within the Great Lakes basin, many of the examined lampreys exhibited different forms of skin lesions that ranged from simple skin erosions, severe skin darkening, deep fin rot and ulcerations. Most of the lampreys were lethargic with remarkable lesions in the eyes and nostrils. Despite the fact that ***Flavobacterium*** psychrophilum is a salmonid specific pathogen, the current study provides the evidence that sea lamprey is a new host range for this pathogen. From the nostrils and skin ulcers of affected lampreys, F. ***psychrophilum*** were

retrieved and further characterized using both conventional biochemical and molecular techniques. Thus, it is highly probable that sea lamprey can play a role in the temporal and spatial spread of ***F. psychrophilum*** to the salmonids residing in the Great Lakes basin.

INTRODUCTION

Diseases caused by ***Flavobacterium psychrophilum*** (***F. psychrophilum***), a gram negative long rod (0.2-0.75 µm in diameter X 1.5-7.5 µm long, has became recently one of the most crucial problems affecting salmonid culture worldwide (**Pacha 1968; Lorenzen et al. 1997**). In North America, the disease caused by F. psychrophilum is known as cold water disease (CWD) because of its occurrence usually at low water temperature among different species of salmonids. The disease is characterized with skin erosions, darkening, and ulcer at the base of fins, gills and caudal peduncle. In severe cases, the caudal fin is completely sloughed with the bare spine and the caudal peduncle area is completely exposed (**Davis 1946; Wood 1974 and Holt et al. 1989**). In very young ages the disease can be associated with nervous manifestation such as erratic swimming behavior and spiral movement (Kent et al.1989). Anorexia, dark skin pigmentation, ascitis and exophthalmia were also reported in early life stages (**Lorenzen et al. 1991 and Bruno 1992**).

F. psychrophilum has been reported from almost all salmonid species (**Cipriano and Holt 2005**). The bacterium has been reported from other non salmonid species such as Ayu, Plecoglossus altivelis (**Lee and Heo**

1998); Carp, Cyprinnus carpio ; Crucian carp, ***Carassius carassius***; Eel, ***Anguilla anguilla***; Tench, Tinca tinca **(Lehmann et al. 1991)**; Fork-tongue goby, ***Chaenogobius urotaenia***; Japanese dace, T***rybolodon hakonensis***; Lake goby, Rhinogobius brunneus **(Amita et al. 2000)**; Pale chub, Zacco platypus **(Iida and Mizokami 1996)**; Perch, ***Perca fluviatilis***; Roach, ***Rutilus rutilus*** **(Madetoja et al. 2002)**. However, the bacterium has never been reported from the sea lamprey or any other agnatha, nor the role played by the sea lamprey in the dissemination of the bacterium to the cohabitant salmonids was previously investigated in the Great Lakes basin.

In the Great Lakes basin, a number of introduced species have invaded the system and caused serious catastrophes to the aquatic habitat **(Lupi and Hoehn 1998)**. Sea lamprey (***Petromyzon marinus***), is one of these invaders which has been counted as 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 early 1940s and 1950s **(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 male sterilization and release to compete with females for spawning. Field assessments indicated a decreased hatch rate in streams where this strategy was practiced. Currently, males are collected from different areas in the Great Lakes basin such as St Marys River at Lake Ontario,

transported into a sterilizing facility in Hammond Bay, Michigan, and then released into selected river systems basin-wide. These procedures of relocation of lampreys may additionally transfer various pathogens including Flavobacterium spp and other existing and non existing fish pathogens, which have raised major concerns regarding the possibility of introducing detrimental infections into virgin or non exposed fish populations at these areas of the Great Lakes.

In the current study, we report isolation of ***F. psychrophilum*** from sea lamprey with various skin lesions. This report is considered the first report of ***F. psychrophilum*** from Agnatha in general and sea lamprey in specific. Data shown in the current study might shed the light on the potential role of the sea lamprey in spreading ***F. psychrophilum*** infection to their cohabitant salmonids in the Great lakes basin.

MATERIALS AND METHODS

Lampreys. 118 adult sea lamprey were transported alive from the Humber River and Duffins Creek, Lake Ontario, to the Aquatic Animal Health Laboratory (AAHL) at Michigan State University for health inspection. The health inspection is performed to determine their suitability for transfer to the lamprey sterilizing facility at Hammond Bay, then into the St. Maryís River, both within the Lake Huron watershed.

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

Bacterial Isolation. Skin lesions were swapped with alcohol 70 % followed by washing several times with PBS (Phosphate Buffered Saline). Bacteriological swaps were taken from the skin and nostrils lesions and spread onto tryptic soy agar (TSA, Remel, Lenexa, Kansas, USA) and Hsu-Shotts agar. Hsu-Shotts agar was prepared by weighing 1 gram of Tryptone powder, 0.25-gram yeast extract, 1.5 gram gelatin and 7.5 gram agar and suspending the dehydrated media into 500 ml sterile distilled water. Mixture was mixed thoroughly, heated with frequent agitation using hot plate magnetic stirrer. Mixture was boiled for 1 minute on the hot plate to completely dissolve the powder then autoclaved at 121°C for 15 minutes. Media was cooled down till 45 °C and 50 µl of filter sterile neomycin sulfate solution were added to the 500 ml media and mixed well before aliquoted into the sterile plates.

Inoculated TSA and Hsu-Shotts plates were incubated at 15°C for up to 5 days. Incubated plates were checked for bacterial colonies growth on a daily basis. Isolates were identified according to the standard morphological and biochemical criteria for ***F. psychrophilum*** described by **(Borg 1960;**

Pacha 1968, Lorenzen et al.1997). Identification tests were subsequently performed on these pure isolates.

Biochemical Identification. Bacterial isolates were preliminarily identified using conventional biochemical tests including: Catalase with 3% hydrogen peroxide solution, cytochrome oxidase with Pathotec oxidase strips (Remel), sugar utilization and H2S production using triple sugar iron (TSI, Remel) , oxidation and fermentation of glucose using OF Basal media with glucose as soul carbohydrate source (DIFCO- BD and Company Sparks, MD, USA), 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 %. Flexiruben pigment production using 20 % potassium hydroxide, gelatin liquefaction using dehydrated nutrient gelatin media (Remel), growth in the presence of 2 % NaCl, growth at 15°C, 30°C and 37°C.

Isolates identified as ***F. psychrophilum*** using these conventional biochemical tests were subsequently analyzed for confirmatory identification using API 20NE tests (BioMerieux). API 20NE tests strips were inoculated per manufactureís instructions incubated at 15°C and interpreted at 48-72 hrs.

Molecular Identification. Chromosomal DNA was extracted from 100 µl bacterial suspension (single colony of each isolated bacteria suspended in

100µl sterile saline) using DNeasy tissue extraction kit (Qiagen-Valencia, CA, USA) according to manufacturerís instructions. The extracted DNA was amplified using oligonucleotide primer set specific for F. psychrophilum described in (Urdaci et al. 1998 and del Cerro et al. 2002). The sequence of the two primers were: primer-1 (5'- CTT AGT TGG CAT CAA CAC -3') and primer-2 (5'- ACA CTG GCA GTC TTG CTA -3'). The controls were composed of a PCR mixture containing no DNA template reagent (negative control) and DNA extracted from a known F. psychrophilum isolate (positive control). A volume of 10 µl of the PCR 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 971 bp band specific for ***F. psychrophilum*** was detected.

RESULTS &DISCUSSION

Clinical examination revealed the presence of skin lesion in Sea Lamprey from Duffins Creek in the form of shallow skin ulcers, erosions in the dorsal and caudal fin (Figure 1 & 2). Some lamprey showed abnormally severe skin darkening. A white slimy film on the nostrils and eyes was also observed on affected lampreys. A total of 118 sea lamprey were analyzed for bacterial pathogens during a routine health inspection for a relocation

procedure during the period of the experiment. ***F. psychrophilum*** was isolated from only 2 out of 118 animal (1.7 % of the samples). One of the ***F. psychrophilum*** isolates was isolated from the white film covering the nostrils and the other one was isolated from the dorsal fin lesion (fin erosion). However, it is crucial to note that, these two positive lampreys were from one collection site, Duffins Creek. This has epidemiological significance because the percent of ***F. psychrophilum*** positive lampreys is then skewed between collection sites; Duffins Creek 3.45 % positive (2 of 58) whilst Humber River was 0% positive.

The two isolates were identified as ***F. psychrophilum*** using both conventional and molecular techniques. On Hsu-Shotts medium, the two isolates showed bright yellow colonies 2- 3 mm in diameter with thin spreading margins (Figure 3). Phenotypically, the two isolates were Gram negative long bacilli, motile by gliding, very weak catalase positive, weak cytochrome oxidase positive, gelatinase test positive, non agarolytic, produces flexiruben upon addition of 20 % KOH (colonies turned into brown orange in color), no growth was observed on the TSA plates at 15°C or 20°C as well as in the presence of 2 % NaCl. The two isolates showed optimal growth at 15°C but there were no growth at 30°C or 37°C. Performing the O/F test using glucose as a sole source of carbon revealed that the two isolates were negative. The two isolates were not able to utilize sucrose, starch, and glucose in their basal media.

Table 1. Results of the phenotypic characteristics and biochemical reactions of the two retrieved for *F. psychrophilum* isolates

Reaction/ Test	Result
Gram reaction	-*
Gelatin hydrolysis	++***
Growth on TSA	-
Agar hydrolysis	-
Catalase	+**
Cytochrome oxidase	+
Starch hydrolysis	-
Sucrose hydrolysis	-
Glucose oxidation	-
Glucose fermentation	-
Gliding motility	++
Growth at 15ºC	+++****
Growth at 30 ºC	-
Growth at 37 ºC	-
Growth in 2.0% NaCl	-
Hydrogen sulfide	-
Indole	-
Nitrate reduction	-
ONPG test	-
Flexirubin pigment	++

* (-) negative ** (+) weak positive *** (+) positive **** (+++) optimal

Figure (1): Sea lamprey showed severe erosion on the ventral aspect of the caudal fin caused by ***F. psychrophilum*** infection. Note the coloration of the margin of the erosion indicating the chronic nature of the lesion.

Figure (2): Sea lamprey showed severe erosion on the dorsal fin caused by ***F. psychrophilum*** infection. Note the coloration of the hemorrhagic margin of the erosion indicating the acute nature of the lesion.

Figure (3): ***Flavobacterium psychrophilum*** colonies isolated from nostrils of Sea lamprey on Hsu-Shotts agar medium.

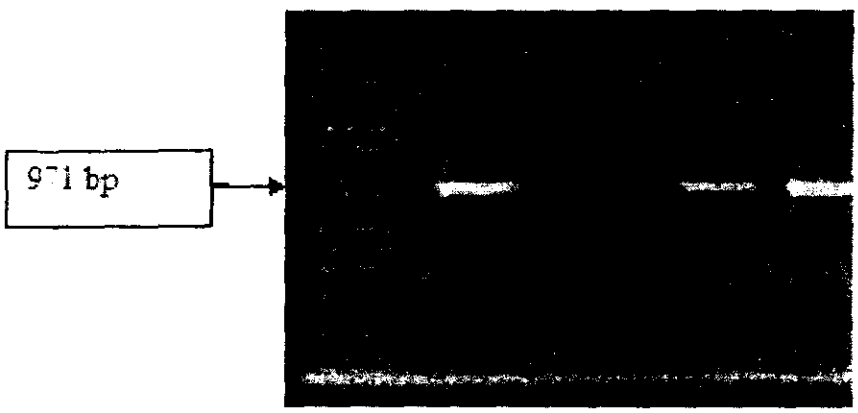

Figure (4): Detection of Flavobacterium psychrophilum infection in Sea Lamprey using PCR according to del Cerro et al 2002.

Lane 1: 100 bp ladder
Lane 2: Positive control of ***F. psychrophilum***
Lane 3: negative control
Lane 4&5: ***F. psychrophilum*** strains isolated from the Sea lamprey

To confirm these preliminary identifications BioMerieux API 20NE rapid test strips were inoculated. The strips were inoculated and interpreted as described in (Table1) and the results were compared to published biochemical reactions (Cipriano and Holt 2005) which confirmed that the two isolates were ***F. psychrophilum.***

Molecular confirmation of the two isolates was done using ***F. psychrophilum*** species specific primer set targeting the 16S rRNA genes **(Urdaci et al. 1998; del Cerro et al. 2002)**. The PCR amplification product of the expected size (971 bp) was observed from both isolates confirming their entity as ***F. psychrophilum*** isolates (Figure 4).

In conclusion, the current study reports a new non salmonid host for ***F. psychrophilum*** infection for the first time in the Great Lakes and worldwide. The affected lampreys showed skin lesions similar to what were reported in salmonids during CWD infection. Furthermore, the long term natural cohabitation between clinically infected parasitic lampreys and salmonids residing in the same water could be a potential for the spread of ***F. psychrophilum*** through the Great Lakes basin fisheries. Despite the broad host range of for ***F. psychrophilum*** in North America, it is difficult to assess the inclusive impact of Lamprey infection on salmonid fish population in the Great Lakes. However to assess the comprehensive impact of for ***F. psychrophilum*** infection on Great Lakes, several factors should to be taken in consideration. Further studies are required to study the molecular similarities of the isolates for ***F. psychrophilum*** strains with those isolated

from salmonids and pathogenicty to salmonids. Unless we reach conclusive evidence of the impacts of this infection, the risk-benefit margins of the lamprey transfer or relocation programs would be uncertain.

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تسجيل أول حالة للعدوى بميكروب الفلافوبكتريم سكيروفيليوم فى اللأمبرى البحرى من بحيرة أونتاريو"

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أثناء الفحص الطبى السنوى لمجموعة من أسماك اللامبرة البحرى من بحيرة أونتاريو ذلك لدراسة قابلية إعادة توزيعهم بالمياه الإقليمية للبحيرات العظمى وجد أن عدد كبير منهم مصاب بإلتهابات جلدية تتراوح بين تاكلات فى طبقة الجلد السطحية ودكانه شديد فى لون الجلد و قرح جلدية شديدة . كذلك فإن الإستجابة العصبية فى معظم أسماك اللامبرى البحرية كانت منخفضة مع وجود بعض الإلتهابات فى العين والأنف وعلى الرغم من أن ميكروب الفلافو بكتريوم سيكروفيليوم مرتبط بأسماك السالمون إلا أن الدراسة تمدنا بدليل فعلى على أن أسماك اللامبرى البحرى تعتبر عائلاً جديداً للعدوى بهذا الميكروب . علماً بأنه قد تم عزل ميكروب الفلافوبكتريوم سيكروفيليوم من الأنف والقرح الجلدية للامبرى المصابة وتم تصنيف الميكروب بإستخدام الطرق البيوكيميائية والبيولوجية الجزيئية الحديثة (PCR) وذلك أدى إلى إحتمال أن يكون للامبرى البحرى دوراً مؤقتاً فى إنتشار العدوى بهذا الميكروب إلى أسماك السلامون المجاورة والمتعايشة معها فى نطاق مياه البحيرات العظمى .