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SHORT COMMUNICATION

Preliminary studies on the isolation of bacteria from sea lice, *Lepeophtheirus salmonis*, infecting farmed salmon in British Columbia, Canada

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Abstract Using standard OIE bacteriological screening protocols, we sampled the external carapace and internal stomach contents of motile stages (preadult and adult) of *Lepeophtheirus salmonis* collected from farmed Atlantic salmon from May 2007 to April 2008 in British Columbia, Canada. Three potentially pathogenic bacteria (*Tenacibaculum maritimum*, *Pseudomonas fluorescens*, and *Vibrio* spp.) were isolated from external (58–100%) and internal (12.5–100%) samples of sea lice. The prevalence of bacteria was higher from lice collected during the months with higher water temperatures and among adult lice. These preliminary results have led to a comprehensive, multi-year study where we plan to examine the possible role of sea lice as a vector for disease.

Introduction

Many terrestrial arthropod ectoparasites are vectors of significant pathogens and their ecology is well documented. For example, *Anopheles* mosquitoes transmit *Plasmodium* malaria, *Ctenocephalides* fleas transmit *Yersinia* plague and *Boophilus (=Rhipicephalus)* ticks transmit *Babesia*. Conversely, studies on aquatic parasites as vectors are primarily descriptive. It has been hypothesized that monogene flukes could be vectors of fish viruses and bacteria (Cusack and Cone 1985, 1986). Similarly, monogene flukes have been reported carrying infectious ectoparasitic flagellates (Colorni 1994) and endoparasitic myxozoans (Aguilar et al. 2004). Leeches have also been documented as vectors of fish blood flagellates and it has been hypothesized that they transmit pathogenic viruses and bacteria (Cusack and Cone 1986; Burreson 2006). Finally, the sea lamprey, *Petromyzon* has been suggested as a vector of *Aeromonas salmonicida* (causative agent of furunculosis) among wild and farmed salmonids (El Morabit et al. 1991).

Farmed salmon in British Columbia (BC), Canada provide a somewhat unique host system to study because of seasonal overlapping distributions of abundant wild salmon co-existing with farmed salmon species (of various life stages) in the region. This situation provides an opportunity to study the potential for pathogen exchange via a possible vector such as sea lice. Two parasitic copepod species ('sea lice'), *Lepeophtheirus salmonis* and *Caligus clemensi* are commonly reported from farmed Atlantic (*Salmo salar*) and wild Pacific (*Oncorhynchus* spp.) salmon (Beamish et al. 2005, 2006, 2007; Saksida et al. 2007a, b) in BC. In addition, the infectious salmon anemia virus (Nylund et al. 1994) and the pathogenic bacteria, *A. salmonicida*, (Nese and Enger 1993) have been isolated from sea lice (*L. salmonis*) infecting clinically diseased fish, thereby implying sea lice could be suitable vectors. Given that sea lice development is affected by salinity and temperature (Johnson and Albright 1991; Brooks 2005; Brooks and Stucchi 2006), seasonal and annual fluctuations in abiotic (salinity, temperature) and biotic (host migration) influences should result in seasonal

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and annual variations in the abundance of any pathogens they carry. Here, we present the results of our pilot study that has led to a multi-year study to examine the role of sea lice as suitable vectors of salmonid pathogens. This document represents the first published isolation of three bacteria, *Tenacibaculum*, *Pseudomonas*, and *Vibrio*, from sea lice parasitizing farmed Atlantic salmon in BC, Canada.

Materials and methods

Sampling Specimens of *L. salmonis* ('sea lice') were collected by our collaborators (Marine Harvest Canada, BC Ministry of Agriculture and Lands) during regular monthly sea lice surveys between May 2007 to April 2008 as part of the Sea Lice Management plan for BC salmon farms (BC MAL 2008). During each survey, our collaborators collected basic water quality parameters (dissolved oxygen, salinity, and temperature) at 1, 5, and 10 m, recorded by an electronic temperature salinity probe (YSI Inc.). For all lice collections, 20 Atlantic salmon, *Salmo salar* (all greater than 6 months in seawater) per netpen were sampled. For this pilot study, our sampling objective was to collect a minimum of 15 lice and maximum of 40; however, if 15 lice were not obtained using the first 20 salmon examined, then an additional 20 fish were sampled from another netpen. We obtained samples from five of the British Columbia Ministry of Agriculture & Lands (BC MAL) fish health surveillance zones (BC MAL 2008) surrounding the coastline of east and west Vancouver Island. Within our sampled zones, >80% farm Atlantic salmon. We collected the motile, actively feeding phases (preadult and adult males, preadult, adult, and gravid females) of *L. salmonis*. In initial samples, we had also examined attached, non-motile chalimus stages, but these were negative for pathogenic bacteria, perhaps as a consequence of their limited feeding activity (Johnson and Albright 1991). As sampling occurred as part of routine sea lice monitoring where fish were anesthetized and not killed for examination, no fish were collected for further bacteriological testing. Moreover, there were no overt signs of disease in any of the fish examined. During the final April 2008 collection, fish were being harvested, allowing seven fish (with lice) to be sampled. For these harvested fish, we performed an aseptic swab of the kidneys only from those fish that we collected lice.

Bacteriology Lice were aseptically removed from salmon using alcohol-sterilized forceps and placed in vials (one vial per host) of filtered, autoclaved (120°C for 40 min) seawater (ASW) and stored for a maximum of 24 h (on ice) prior to screening. From each louse, we sampled the exoskeleton by using one sterile cotton-tipped swab from the dorsal and ventral surfaces of the cephalothorax. Next, each specimen was disinfected with 70% ethanol for 10 s and rinsed in ASW prior to collecting an internal sample. As a verification of external disinfection, we collected a second external swab from the lice for plate inoculation. From experience, this disinfection technique was quite successful and the disinfection was not excessively long such that the alcohol sterilized internal stomach contents. For internal lice samples, we aseptically (alcohol-flamed scalpel) opened the ventral surface of the cephalothorax, abdomen, and stomach, then swabbed the stomach contents using a sterile, disposable, plastic inoculating loop. We also sampled 0.5 ml of the ASW that contained the sampled lice. For primary screening, all swabbed samples (from lice or salmon) were inoculated on a variety of nutrient media: Marine agar (MA), Tyes Agar (TA) + 3% sea salt (both at 22°C) and blood agar (BA) + 3% sea salt, brain heart infusion (BHI) agar + 3% sea salt (both at 12°C) for a maximum of 7 days. From these primary cultures, pure subcultures were isolated, Gram-stained, then identified by simultaneously using a variety of differential media and biochemical assays: oxidative–fermentative (O-F) media, motility media, oxidase, 2% KOH test, API-20E/20NE (verified by apiweb®) and vibriostatic ($0/129_{150}$) sensitivity disks.

Results and discussion

The prevalence of infection ranged from 30–55% with an intensity of 0.8–2.7 lice per fish. Most of the lice examined were non-motile (chalimus) stages. We obtained sufficient numbers of lice (minimum of 15 from 20 or 40 salmon/farm) to sample only during the months of May, June, August, December, and April, with multiple samples from different sites during May and June. From these samples, we isolated three bacterial species *Tenacibaculum maritimum, Pseudomonas fluorescens*, and *Vibrio* spp. (Table 1) from both external (58–100%, Table 2) and internal (12.5–100%, Table 2) samples of sea lice. Most bacteria were isolated from external sampling, likely associated with surface contamination from pooled samples per fish. We were able to isolate bacteria from internal gut contents of lice that had been previously sterilized externally. Sexually mature lice had a higher prevalence of infection than the preadult stages (90–100% vs. <40%, respectively). The observed prevalences of bacteria were higher among male than female lice in the spring; whereas in the summer and late autumn, observed prevalences were higher among female lice (Table 3). These observations did not correlate with varying sex ratios among these months. Prevalence of bacteria was highest during the summer months when

Table 1 Morphology and biochemistry profiles of *Tenacibaculum maritimum*, *Pseudomonas fluorescens* and *Vibrio* spp from motile stages of sea lice, *Lepeophtheirus salmonis*, parasitizing healthy farmed Atlantic salmon in B.C., Canada

Variable	*Tenacibaculum maritimum*	*Pseudomonas fluorescens*	*Vibrio* spp.[a]
Colony features diameter (mm)	Pale yellow, dry, flat, rhizoid, (3.0–10.0+)	Yellow, fluoresces diffusible (3.0–6.0)	Cream, smooth, (2.0–3.0)
Growth media	MA, Tyes + 3% salt	MA, BA + 3% sea salt	MA, BA + 3% sea salt
Gram size (W × L, μm)	(−) Long bacillus, (0.5×2.0–28.0+)	(−) Straight bacillus, (0.5×1.4–3.5)	(−) Curved bacillus, (0.5–1.0×1.7–2.5)
Oxidase	+	+	+
Motility	(−) gliding	+	+
$0/129_{150}$	−	−	+
Oxidative/fermentative	+ / −	+ / −	+ / +
Nitrate Reduction	−	−	+
Arginine dihydrolase	−	+	Variable
Ornithine decarboxylase	−	−	−
Urease	−	−	−
Beta-galactosidase	−	−	−
Citrate utilization	−	+	Variable
H_2S production	−	−	−
Urease	−	−	−
Indole production	−	−	Variable
Acetoin (VP)	−	−	−
Gelatinase	+	+	+
Acid from glucose	−	(+) Weak	+
Acid from mannitol	−	+	+
Acid from inositol	−	+	−
Acid from sorbitol	−	+	−
Acid from rhamnose	−	−	−
Acid from melibiose	−	−	−
Acid from arabinose	−	+	−

[a] *Vibrio* species mixed and identified as *V. alginolyticus* and *V. vulnificus*

Table 2 Average water temperature and salinity with monthly prevalence (%) of *Tenacibaculum maritimum*, *Pseudomonas fluorescens*, and *Vibrio* spp. from external (EX.) and internal (INT.) samples of motile stages of sea lice, *Lepeophtheirus salmonis*, parasitizing healthy farmed Atlantic salmon in BC, Canada collected from various fish health surveillance zones (Z) during 2007 and 2008

Sample sizes of lice listed with each BC MAL surveillance zone (Z).

[a] *Vibrio* species mixed and identified as *V. alginolyticus* and *V. vulnificus*

Bacteria	Temp.	Salinity	*T. maritimum*		*P. fluorescens*		*Vibrio* spp.[a]	
	°C @ 1m	‰	EX.	INT.	EX.	INT.	EX.	INT.
Month								
May 2007								
Z-2.3, n=23	10.2±0.6	26.3±0.6	100%	30.4%	60.8%	73.9%	–	–
Z-3.2, n=26	9.2±0.4	31.3±0.4	100%	15.3%	57.6%	73.1%	–	–
June 2007								
Z-2.4, n=15	10.8±0.5	31.1±0.3	100%	100%	100%	86.7%	–	–
Z-3.3, n=15	9.8±0.6	33.3±0.6	100%	93.3%	100%	93.3%	–	–
Z-3.4, n=21	11.8±0.6	22.5±2.1	100%	19%	100%	19%	–	–
August 2007								
Z-2.3, n=40	14.2±0.4	31.3±0.9	100%	35%	100%	15%	100%	25%
December 2007								
Z-3.3, n=40	7.8±0.3	30.1±1.6	100%	27.5%	–	–	–	–
April 2008								
(*Z*-3.3, n=20)	8.8±0.6	27.3±2.6	100%	35%	50%	30%	–	–

Table 3 Monthly prevalence (%) of *Tenacibaculum maritimum* from external and internal samples of male (M) and female (F) motile (preadult and adult) sea lice, *Lepeophtheirus salmonis*, parasitizing farmed Atlantic salmon in BC, Canada during 2007 and 2008

Sample sizes of lice in parentheses after each BC MAL surveillance zone

		Sex ratio%	*T. maritimum*	
		M:F	M	F
Month				
May 2007	(Z-2.3, n=23)	48:52	18%	8.3%
	(Z-3.2, n=26)	27:73	28%	26%
June 2007	(Z-2.4, n=15)	60:40	100%	100%
	(Z-3.3, n=15)	40:60	100%	100%
	(Z-3.4, n=21)	43:57	0%	50%
August 2007	(Z-2.3, n=40)	43:57	23%	48%
December 2007	(Z-3.3, n=40)	43:57	0%	21%
April 2008	(Z-3.3, n=20)	35:65	71%	62%

recorded seawater temperatures were at their highest (10–14°C, Table 2) but there was no apparent association with water salinity.

T. maritimum and *P. fluorescens* were the most common species isolated from external and internal samples from all zones sampled; whereas *Vibrio* was cultured from lice collected in only one area and only during 1 month (August 2007; Table 2). *Tenacibaculum* grew well on the TA + 3% sea salt (3–4 days) and the MA (5–7 days), exhibiting the typical flat, yellow colony characteristics (positive reaction with 2% KOH, indicative of flexirubrin-type pigments) and filamentous cell characteristics (Table 1), but had erratic to low growth on the BA and BHI media. This species is common in marine environments, exhibiting a wide geographic distribution, variable strains, and opportunistic pathogenicity (Austin and Austin 2007, Toranzo et al. 2005), thus, its isolation was not unexpected. *Tenacibaculum* has been locally reported (Ostland et al. 1999, BC MAL 2008) from farmed salmon in BC as a causative agent of fin and mouth rot, 'myxobacterial stomatitis' especially among newly transferred fish (e.g., <6 months in seawater). *Tenacibaculum* adheres to fish mucous (Austin and Austin 2007); thus, the potential for an overlap in occurrence with sea lice exists. Conversely, a somewhat mutually exclusive occurrence of sea lice (mostly in fish >6 months in seawater) and *Tenacibaculum* (mostly in fish <6 months in seawater) should theoretically prevent/minimize any type of overlapping occurrences. However, in our April 2008 sample, we also isolated *Tenacibaculum* from a kidney swab of the same adult salmon hosts (n=7) that we obtained our lice with *Tenacibaculum* detected in their stomach contents. Furthermore, historical farm health records indicate that *Tenacibaculum* infections have been isolated from adult salmon (>6 months in seawater) collected within our sampled zones (Coombs and Boyce, pers. comm.). *Tenacibaculum* does not survive well in seawater because is easily out-competed by other bacterial species (Avendaño-Herrera et al. 2006); thus, in our next study, we will test if the presence of lice provides suitable (but likely ephemeral) organic substrate for *Tenacibaculum* which can extend its longevity in seawater.

Although less commonly isolated from our samples, *Pseudomonas* and *Vibrio* both grew well on the MA (2 days) and BA + 3% sea salt (2–3 days) plates at both temperatures (Table 1). *Pseudomonas* is described as a secondary invader, also associated with fin rot and variable pathogenecity among salmonids (Austin and Austin 2007; Sakai et al. 1989). *P. fluorescens* is often associated with infections in freshwater; however, one of us (M.P.C.) had also isolated *P. fluorescens* in May 2007 from the farmed Atlantic salmon held in the same netpen from which we obtained our sea lice in June 2007.

Our isolates of *Vibrio* were equivocal in that our biochemical assays resulted in different species (*V. alginolyticus* and *V. vulnificus*) from the same sub-cultures. *Vibrio* spp. (pathogenic and non-pathogenic) are widespread in the marine environment. *Vibrio vulnificus* has traditionally been categorized as a bacterial pathogen of eels, but has been reported (Austin and Austin 2007; Lunder et al. 1995; Toranzo et al. 2005) from other marine fishes, including salmonids. Interestingly, Hansen and Bech (1996) isolated similar bacteria genera (*Vibrio* spp., *Pseudomonas* spp. and a *Tenacibaculum*-like, *Cytophaga/Flavobacterium* spp.) from free-living marine copepods, *Acartiatonsa*, held under experimental conditions.

Our pilot study has documented isolation of *Tenacibaculum*, *Pseudomonas*, and *Vibrio* from the external surface and stomach contents of sea lice parasitizing farmed salmon. It should be expected that an epidermal grazing fish ectoparasite such as sea lice will encounter obligate pathogens from the fish's skin. The next logical step would be to determine if *L. salmonis* can act as a vector to spread disease within a population or between populations (e.g., between wild/farmed). Therefore, we have begun a multi-year study to explore this concept by first testing transmission from experimentally infected salmon hosts to naïve sea lice and vice-versa. In our studies, we hope to define whether the lice act serendipitously as a mechanical

carrier (source of accidental transport only), or whether the lice truly fulfill the role of a biological vector (source of pathogen development and transmission). Given the different swimming behaviors and host range of *Lepeophtheirus* and *Caligus* we will also examine any differences (in terms of vector potential) among these key ectoparasitic species.

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