Modes of transmission of *Loma salmonae* (Microsporidia)

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ABSTRACT: *Loma salmonae* (Putz, Hoffman and Dunbar, 1965) Morrison and Sprague, 1981 (Microsporidia) causes prominent gill disease in pen-reared chinook salmon *Oncorhynchus tshawytscha* in the Pacific Northwest. Transmission of the parasite was examined by exposing Pacific salmon *Oncorhynchus* spp. to infectious spores by various routes: *per os*, intraperitoneal, intramuscular, and intravascular injection, by cohabitation with infected fish, and by placement of spores directly on the gill. All exposure methods led to infections except placement of spores on the gill. Putative sporoplasms were visible in epithelial cells of the alimentary canal within 24 h of *per os* exposure. *L. salmonae* may initially infect alimentary epithelial cells and then migrate into the lamina propria to access the blood stream. Positive results obtained by intravascular injection suggest that autoinfection from spores of ruptured xenomas in the endothelium may also occur. The cohabitation experiment demonstrates that fish may become infected by spores released from live fish.

KEY WORDS: *Loma salmonae* Microsporidia Transmission

INTRODUCTION

Loma salmonae (Putz, Hoffman and Dunbar, 1965) Morrison and Sprague, 1981 is a microsporidian parasite infecting endothelial cells of salmonids. Fish infected with *L. salmonae* often exhibit pale gills with petechial hemorrhages, inflammation, hyperplasia, and white cysts termed xenomas (Wales & Wolf 1955, Hauck 1984, Kent 1992). Outbreaks of *L. salmonae* in Pacific salmon *Oncorhynchus* spp. and rainbow trout *O. mykiss* have occurred in the Pacific Northwest (Hauck 1984, Kent et al. 1989), eastern United States (Markey et al. 1994), and Scotland (Bruno et al. 1995).

Kent et al. (1995) demonstrated that fish can be infected by *Loma salmonae* when ingesting infected tissues. Whereas details on the early development of *Loma* species are unknown, for microsporidia in general the spore extrudes a polar filament which pierces a gut epithelial cell and injects the parasite's sporoplasm. For genera such as *Loma* and *Glugea*, the

sporoplasm eventually divides, forming numerous spores within a hypertrophied cell (i.e. a xenoma) (Canning & Lom 1986).

Loma salmonae has been transmitted experimentally both in fresh and sea water by feeding fish macerated gills containing spores (Kent et al. 1995). Hauck (1984) suggested that gills may be infected directly by phagocytic uptake of pillar cells. However, the method by which *Loma* spp. spread within the host is unknown. It may be spread by infected macrophages, or by utilizing body fluids (Hauck 1984). In the present study, experimental transmission of *L. salmonae* to chinook *Oncorhynchus tshawytscha* and coho *O. kisutch* was attempted by various routes: *per os*, intraperitoneal (IP), intramuscular (IM), and intravascular (IV) injection, cohabitation of infected fish with naive fish, and placement of *L. salmonae* spores directly on the gills.

MATERIALS AND METHODS

Fish husbandry. Fish were obtained from Rosewall Creek Hatchery, Fisheries and Oceans Canada, Van-

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couver Island, Canada. This hatchery receives only well water and has no history of the disease. Unless noted elsewhere, all fish were held in flow-through tanks receiving 14 to 16°C dechlorinated fresh water. Before handling, fish were anaesthetised with tricaine methanesulfonate (MS-222). During sampling fish were killed with an overdose of MS-222; the first left gill arch of each fish was examined by wet mount for *Loma salmonae*. The rest of the gills were placed in Davidson's solution (Humason 1979) along with the heart, stomach, intestine, kidney, liver, and spleen, and a section of dorsal muscle for histology. All these tissues were processed using standard histological techniques. Experiments were terminated at 56 d, unless otherwise noted, to allow sufficient time for development of visible xenomas (Kent et al. 1995).

Preparation of parasite. Spores of *Loma salmonae* were purified from infected chinook gills obtained from a seawater netpen site on the west coast of Vancouver Island. Gill tissue suspended in dechlorinated fresh water was ground using a Polytron tissue homogeniser (Luzer, Switzerland) to create a slurry. This slurry was centrifuged at $2000 \times g$ for 10 min. The pellet was resuspended in water and filtered through a wire mesh and then through a 50 μ m nylon screen. The filtrate was then centrifuged at $800 \times g$ for 45 min on a layered 34%/51% Percol gradient. Pure spores were collected from the pellet and the 34%/51% layer.

Exposure protocol. *Loma salmonae* spores were introduced directly into the stomach using a 16 g needle tipped with 2.0 mm inner diameter tubing as either a gill tissue slurry (0.25 ml) or as a suspension of pure spores (0.1 ml). For anal gavage, a 22 g needle tipped with 0.58 mm inner diameter tubing was used. Various amounts of pure spores (Tables 1 & 2) in 0.1 ml inocula were used for anal gavage, IP, IM, and IV injections. IV injections were given using the ventral gill sinus or dorsal aorta.

Various trials were conducted using the above exposure methods as outlined in Tables 1 & 2. Controls had their left ventral fin clipped and were kept in the same tank as exposed fish. In trial 6, controls were injected with Earle's Buffered Saline Solution (EBSS) and blood smears were collected at all sampling times from fish receiving IV injections. For trial 3, blood smears from 3 fish were made within 30 min of injection. Smears were air dried, heat and methanol fixed, and an indirect fluorescent antibody test (IFAT) was conducted to locate spores. Monoclonal antibody (MAB) for *Loma salmonae* developed using standard techniques (Schots et al. 1992) was provided by F. Markham, Department of Pathology and Microbiology, Atlantic Veterinary School, Charlottetown, Prince Edward Island, Canada. The MAB (225 μ l) was applied directly to blood smears and allowed to incubate at room temperature for

30 min. The smears were washed 3 times and incubated in phosphate buffered saline (PBS) at pH 7.2 for 10 min. Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG antibody diluted in PBS (1:128) was counterstained with 1% Evan's blue (1:156) and applied to the smears. Smears were incubated for 30 min, washed 3 times and incubated for 10 min in PBS. IFAT slides were examined with a fluorescent microscope at $\times 1000$ using pH 9 mounting fluid (Difco).

In trial 4, pure spores (0.1 ml) were placed directly on the left gill arch of 10 chinook held out of water for 1 min. After 1 min, each chinook was dipped for 5 s in a rinse bucket before being placed in the holding tank. Controls were passed through the rinse bucket before

Table 1 *Loma salmonae* in *Oncorhynchus tshawytscha* and *O. kisutch*. Infection of *L. salmonae* in chinook and coho salmon exposed by various routes. Fish were examined at 56 d post-exposure and considered positive upon detection of the parasite in wet mounts or histological sections

Trial	No. of fish exposed	No. positive/ No. examined
Trial 1		
Chinook (avg. 16.6 cm; 38.4 g)		
Spore dosage (except controls) = 2.8×10^6		
Per os	10	9/10
Anal gavage	10	10/10
Control	4	0/4
Trial 2		
Coho (avg. 18.8 cm; 60.1 g)		
Spore dosage = 1.2×10^6		
Per os	10	9/9
Intraperitoneal	10	8/8
Intravascular ^d	10	10/10
Trial 3		
Chinook (avg. 23.2 cm; 119.2 g)		
Spore dosage (except controls) = 5.9×10^5		
Per os	4	4/4
Intravascular ^{b,c}	13	10/10
Control	4	0/4
Trial 4		
Chinook (avg. 11.2 cm; 12.9 g)		
Spore dosage (except controls) = 2.8×10^6		
Gill arch	10	0/10
Control	2	0/2
Trial 5		
Cohabitation exposure		
Donor chinook (avg. 19.8 cm; 66.4 g)		
Recipient chinook (avg. 12.4 cm; 17.4 g)		
Donor fish (spore dosage = 1.25×10^6)	10	10/10
Recipient fish, 24 h exposure ^d	45	3/10
Recipient fish, 3 d exposure ^d	35	7/10
Recipient fish, 5 d exposure ^d	25	6/10
Recipient fish, 7 d exposure ^d	15	10/10
^a Injection by ventral gill sinus		
^b Injection by dorsal aorta		
^c Spores detected in blood by an indirect fluorescent antibody test (IFAT)		
^d Fish removed at time indicated and raised separately for 56 d before examination		

being placed in the same tank. The same inoculum was used here as in trial 1.

For trial 5, donor chinook were infected *per os* and held for 72 d at 12 to 14°C. Ten donor fish with clinical signs of *Loma salmonae* (anemia and petechial hemorrhages on gills) were placed in a 204 l tank with 45 naive chinook (differentiated by size from infected fish). After exposure periods, sets of 10 recipient fish were removed (Table 1). Following 7 d cohabitation (i.e. 79 d post-exposure of donor fish), all donor fish were examined for *L. salmonae*. Intensity of infection in donors was measured from the average of 3 counts of the number of xenomas per $\times 100$ field of view (1.5 mm diameter).

RESULTS

All exposure methods, except placement of *Loma salmonae* spores directly on the gill, resulted in infection (Tables 1 & 2). When administered *per os*, spores were found within 6.5 h in histological sections of stomach in free material and in association with epithelial cells (Figs. 1 & 2). Between 2.5 and 24 h possible sporoplasms were found in epithelial cells of the stomach and pyloric caeca. These stages contained dark-staining, probably nuclear, material and a lighter-staining outer area. At 24 h epithelial cells of the anterior intestine contained these sporoplasms, and some sporoplasms were also associated with the lamina propria (Figs. 3–5). These structures were not found in controls or in fish sampled at 35 or 56 d.

Loma salmonae spores were detected in the blood by IFAT after fish received IV injections via the dorsal aorta and up to 24 h after injections via the ventral gill

Table 2. *Loma salmonae* in *Oncorhynchus kisutch*. Infection of *L. salmonae* in coho salmon from trial 6 exposed by various routes to 1.2×10^6 spores per fish (except controls). Fish were considered positive upon detection of the parasite in wet mounts or histological sections

Mode of exposure	No. of fish	Time examined	No. positive/ No. examined
<i>Per os</i>	16	2.5 h ^d	0/2
		6.5 h ^{a, b}	0/2
		24 h ^d	0/2
		35 d	1/2
		56 d	7/8
Control	8	2.5 h–56 d	0/8
Intraperitoneal	16	2.5 h	0/2
		6.5 h	0/2
		24 h	0/2
		35 d	0/2
		56 d	3/4
Control	4	2.5 h–56 d	0/4
Intravascular ^c	16	2.5 h ^d	0/2
		6.5 h ^d	0/2
		24 h ^d	0/2
		35 d	1/2
		56 d	6/6
Control	8	2.5 h–56 d	0/8
Intramuscular	16	2.5 h	0/2
		6.5 h	0/2
		24 h	0/2
		35 d	1/2
		56 d	6/7
Control	8	2.5 h–56 d	0/7

^aPossible sporoplasms detected in alimentary canal

^bSpores detected in histological sections of stomach

^cInjection by ventral gill sinus

^dSpores detected in blood by IFAT

Figs. 1 & 2. *Loma salmonae* in *Oncorhynchus kisutch*. Hematoxylin & eosin sections of *L. salmonae* spores in coho salmon infected *per os* and sampled within 6.5 h. Fig. 1. Spores in stomach material denoted by arrows. Scale bar = 12 μ m. Fig. 2. Single spore in association with stomach epithelial cells. Scale bar = 12 μ m

sinus (Fig. 6). By 56 d all fish given IP injections had swollen kidneys and spleens, petechial hemorrhaging, and bloody ascites. This was not seen in fish infected *per os* or in controls. One fish given an IV injection showed edema and hemorrhaging. Fish receiving IM injections of spores exhibited edema and a pronounced inflammatory response in muscle tissue at the site of injection by 35 d (infection shown after 56 d in Fig. 7). No inflammatory changes were seen in controls. In trial 6 (Table 2) xenomas were first detected 35 d post-infection in the gills, heart and spleen. Xenomas were found most often in the gills followed by heart and spleen (Table 3). Xenomas were not found in intestine, liver, muscle, or stomach.

Xenomas in donor fish (Table 1; trial 5) were completely opaque and filled with spores. Intensity in donor fish was 2.32 xenomas (range 1 to 6.3) per $\times 100$ field of view.

Figs. 3 to 5. *Loma salmonae* in *Oncorhynchus kisutch*. Hematoxylin & eosin sections of possible sporoplasms (denoted by arrows) of *L. salmonae* in anterior intestinal epithelial cells of coho salmon infected *per os* and sampled within 24 h. Fig. 3. Sporoplasm in intestinal cell. Scale bar = 6 µm. Figs. 4 & 5. Sporoplasms in association with lamina propria of intestinal cells. Scale bar = 6 µm

DISCUSSION

Oncorhynchus spp. were susceptible to *Loma salmonae* administered by *per os*, anal gavage, IP, IM, and IV injection, and by cohabitation with infected fish. Microsporidia such as *Glugea*, *Heterosporis*, *Microsporidium*, and *Pleistophora* that infect fish species have been successfully experimentally transmitted by *per os*, IP, and IM injection, and by water-borne exposure (Lom 1969, Awakura 1974, McVicar 1975, Leiro et al. 1994). Water-borne infections can result from direct immersion in a spore solution (Awakura 1974, T'sui et al. 1988), or by feeding a crustacean, previously exposed to spores, to fish (Awakura 1974, Olson 1976, 1981). *L. salmonae* has been transmitted *per os* to chinook (Kent & Dawe 1994) and rainbow trout *O. mykiss* (Speare et al. 1998a). In addition Speare et al. (1998b) transmitted *L. salmonae* *per os* and by IP injection of

semi-purified spores to rainbow trout. Hauck (1984) suggested phagocytic uptake of *Loma* sp. by gill pillar cells as water passes through the gills. Our findings do not support this hypothesis as spores placed directly on gills did not result in infection.

Association of *Loma salmonae* spores with stomach epithelial cells, and possible sporoplasms released from spores located within epithelial cells of the stomach, pyloric caeca, and anterior intestine, most likely indicate initial sites of infection. Spores discharging polar filaments may pierce gut epithelial cells, injecting infective sporoplasms. These could migrate and enter the lamina propria, which is richly supplied with blood capillaries. It is generally believed that microsporidia that infect fish enter gut epithelial cells, where they develop or are then transported to their preferred site of development (Canning & Lom 1986). These microsporidia might infect migratory cells such as histio-

Fig. 6. *Loma salmonae* in *Oncorhynchus kisutch*. *L. salmonae* spores detected by IFAT in the blood of coho salmon within 24 h of fish being injected with pure spores via the ventral gill sinus. Scale bar = 12 µm

Fig. 7. *Loma salmonae* in *Oncorhynchus kisutch*. Pronounced inflammatory response in coho salmon muscle after 56 d in association with intramuscular injection of *L. salmonae* spores. Hematoxylin & eosin. Scale bar = 6 µm

Table 3. *Loma salmonae* in *Oncorhynchus kisutch*. Distribution of *L. salmonae* in organs of coho salmon from trials 2 and 6 combined, at 56 d after exposure by various routes. Values for tissue are percentage of fish showing infection in organ indicated

Infection method	No. of fish	Tissue			
		Gill	Heart	Spleen	Kidney
<i>Per os</i>	17	100.0	22.2	17.6	0.0
Intraperitoneal	12	92.2	41.7	41.7	25.0
Intravascular	16	100.0	43.8	41.2	6.3
Intramuscular	7	85.7	14.3	0.0	0.0

cytes, neutrophils or macrophages (Weissenberg 1968, McVicar 1975, Canning & Lom 1986). Matthews & Matthews (1980) proposed that macrophages act as a transport mechanism for *Tetramicra brevifilum* Matthews & Matthews, 1980 through the endothelium of turbot *Scophthalmus maximus*, or become infected themselves during phagocytosis of the parasite in the lamina propria of the intestine. However, to our knowledge none of these hypotheses have been previously experimentally demonstrated. Further studies using *in situ* hybridisation will probably elucidate the site of initial infection and how subsequent spread of *L. salmonae* occurs within the fish host. Docker et al. (1997) developed a polymerase chain reaction (PCR) test for *L. salmonae* which would be useful for this purpose.

Autoinfection within fish-infecting microsporidia has also been proposed but not verified (Lom & Dyková 1992). The systemic distribution of *Loma* sp. xenomas and free spores suggests that, as xenomas rupture, infective stages are liberated and move throughout the fish via the blood (Hauck 1984, Markey et al. 1994). Our experiments, in which free spores injected into the blood resulted in xenoma formation in the gills support the hypothesis that autoinfection of *L. salmonae* may occur, i.e. in natural infections, xenomas may rupture in blood vessels and free spores may circulate and establish new xenomas. The high concentration of xenomas in the gills compared to other organs, regardless of the route of exposure, suggests that the gill endothelium is the preferred site of development of *L. salmonae*, rather than a coincidental site of infection due to route of exposure or circulatory patterns.

Transmission of *Loma salmonae* from infected to naive fish within 24 h of cohabitation in a flow-through system indicates how readily the parasite may be transmitted. Kano et al. (1982) noted that *Heterosporis anguillarum* Hoshina, 1951 (syn. *Pleistophora anguillarum*) spread to healthy eels from infected eels in the same aquarium. During cohabitation in our study, naive fish may have ingested spores from ruptured or whole xenomas released from the gills (i.e. the secondary lamellae) of infected fish. Spores could also be

liberated in fish urine when fish have kidney infections (Hauck 1984).

Our results demonstrate that *Loma salmonae* is transmissible by experimental (e.g. IP, IM) and natural (e.g. *per os*, cohabitation) exposure routes. Infection by IV injection and distribution of xenomas throughout vascular tissue suggest that once a fish is infected, *L. salmonae* may spread within the host by autoinfection. Kent et al. (1995) proposed that *L. salmonae* spreads within a seawater netpen site via spores released from decomposing fish or by other fish feeding on the remains of mortalities. Salmonid farmers should be aware of this and the possibility of live infected fish transmitting the parasite to naive fish when formulating management strategies to control the infection.

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