

# Sensitive and specific polymerase chain reaction assay for detection of *Loma salmonae* (Microsporea)

Margaret F. Docker<sup>1</sup>, Robert H. Devlin<sup>1</sup>, Jon Richard<sup>2</sup>, Jaswinder Khattri<sup>1</sup>, Michael L. Kent<sup>2,\*</sup>

<sup>1</sup>West Vancouver Laboratory, Department of Fisheries and Oceans, 4160 Marine Drive, West Vancouver, British Columbia, Canada V7V 1N6

<sup>2</sup>Pacific Biological Station, Department of Fisheries and Oceans, Nanaimo, British Columbia, Canada V9R 5K6

**ABSTRACT:** Ribosomal DNA sequence of the microsporidian parasite *Loma salmonae*, including portions of the small subunit (SSU), large subunit (LSU), and internal transcribed spacer regions (ITS), was determined. Based on *L. salmonae*-specific regions of sequence, a polymerase chain reaction (PCR) assay was developed for detection of this parasite in chinook salmon *Oncorhynchus tshawytscha*. The specificity of the assay was verified by the lack of amplification with DNA from 3 other microsporidians that parasitize fishes in British Columbia, Canada. A dilution study assessed the sensitivity of the test, showing that the PCR product (derived from multiple copies of rDNA per cell) was routinely detected from as few as 0.01 spores per 50 µl reaction (or 40 spores g<sup>-1</sup> of infected gill tissue). The assay detected *L. salmonae* infections in the gills, kidneys, and ovaries of broodstock females from a seawater netpen on Vancouver Island, British Columbia, and infections in these organs were confirmed by histology. This is the first report of *L. salmonae* infections in ovarian tissue. Although neither PCR nor histology detected *L. salmonae* within the eggs themselves, it is possible that the progeny of infected females may become exposed to the parasite through contaminated ovarian fluid.

**KEY WORDS:** *Loma salmonae* · Microsporidia · Ribosomal DNA · Polymerase chain reaction

## INTRODUCTION

*Loma salmonae* (Microsporea) is an obligate, intracellular parasite which infects the gills and other vascularized tissues of salmonids of the genus *Oncorhynchus* from fresh water throughout the Pacific Northwest of North America (Wales & Wolf 1955, Putz et al. 1965, Morrison & Sprague 1981c, 1983, Hauck 1984, Magor 1987). Similar, if not identical, species have also been reported in several *Oncorhynchus* species from the eastern United States (Markey et al. 1994), Great Britain (Poynton 1986), France (Bekhti & Bouix 1985), and Japan (Awakura et al. 1982). The infection can persist after fish are transferred to sea water (Wood 1974). In fact, the pathological changes associated with *L. salmonae* infection may be more

severe in salmonids in sea water (Kent et al. 1989), and the parasite can easily be transmitted from fish to fish while in sea water (Kent et al. 1995). Consequently, *L. salmonae* has been a serious problem in pen-reared chinook *Oncorhynchus tshawytscha* and coho salmon *O. kisutch* in the Pacific Northwest (Kent et al. 1989, Speare et al. 1989, Kent 1992).

Although preliminary experiments suggest that an antimicrobial agent, Fumagillin DCH, may be a useful therapeutic agent for controlling *Loma salmonae* (Kent & Dawe 1994), no drugs are commercially available for treatment against this parasite. At present, this disease is best controlled by avoiding the spread of infection. The main problem facing this strategy, however, is determining the source of infection. Although the disease can originate in fresh water, a high prevalence of *L. salmonae* infection was observed in chinook from 1 netpen that had been reared solely on ground water during the freshwater phase (Kent et al. 1995). Vertical

\* Addressee for correspondence. E-mail: kentm@pbs.dfo.ca

transmission, from infected females directly into the eggs, or seawater horizontal transmission from a non-salmonid fish may also be sources of infection. The spores of other fish microsporidians have been found in the mature ova of the host (Vaney & Conte 1901, Summerfelt & Warner 1970) and, although *L. salmonae* has only been reported from salmonids, very similar *Loma* species have been reported from strictly marine fishes (Morrison & Sprague 1981a, b).

Studies on the mode of transmission of *Loma salmonae*, and the application of routine screening to limit its spread in netpens, have been limited by the ability of histological analysis to detect infection. Fish may be infected for several weeks, if not months, before the parasite can be observed in histological sections, and this method often lacks sensitivity. The purpose of this study, therefore, was to develop a sensitive and specific DNA-based assay for the detection of *L. salmonae* in chinook salmon. *L. salmonae* ribosomal DNA (rDNA), comprising portions of the small subunit (SSU) and large subunit (LSU) genes and the internal transcribed spacer (ITS) region, was sequenced. Using species-specific regions of this sequence, we developed a polymerase chain reaction (PCR) test that can detect very small amounts of *L. salmonae* DNA, even when mixed with a very large amount of host DNA.

## MATERIALS AND METHODS

***Loma salmonae* ribosomal DNA sequence. Specimen collection:** For the initial isolation of *Loma salmonae* for DNA extraction and sequencing, chinook salmon were collected in March 1995 from a seawater netpen on the west coast of Vancouver Island with a history of the infection. Gills showing infection by *L. salmonae* were frozen at  $-70^{\circ}\text{C}$ . Control (uninfected) chinook salmon were from the Rosewall Creek Hatchery, Fisheries and Oceans Canada, Vancouver Island, Canada, which receives only pure well water and has no history of the disease. Subsamples of tissues were also preserved in Davidson's solution for histological verification of infection. These latter samples were processed for light microscopy using standard histological techniques (Humason 1979), and the sections were stained with Harris' hematoxylin and eosin.

**Spore isolation:** Tissue from heavily infected gills were scraped into Earl's Buffered Saline Solution (EBSS), and centrifuged at  $1200 \times g$  for 10 min. The pellet was resuspended in EBSS, homogenized at low speed, and centrifugation and resuspension were repeated. Large pieces of tissue debris were removed by successive filtration through a wire mesh and then a  $50 \mu\text{m}$  nylon screen. The filtrate was layered onto a

34/51% Percoll gradient and centrifuged at  $500 \times g$  for 45 to 60 min. The layers and pellet were removed separately, concentrated by a further 10 min centrifugation at  $1200 \times g$ , and the spores were stored at  $-70^{\circ}\text{C}$ . Throughout the process, visual inspection verified the presence of the intact spores at each step.

**DNA isolation and PCR:** Host and parasite DNA were extracted from ca 30 mg *Loma salmonae*-infected gills by shaking with 0.5 mm silica beads in TE buffer (10 mM tris, 1 mM EDTA), pH 8.0, for 3 min in a Mini-Beadbeater (Biospec Products, Bartlesville, OK, USA). The suspension was extracted twice with phenol chloroform, and the DNA precipitated on ice with sodium acetate and ethanol. The DNA was resuspended in TE, quantitated, and stored at  $4^{\circ}\text{C}$ . DNA was similarly extracted from ca 40  $\mu\text{l}$  (packed volume) of purified *L. salmonae* spores, although  $1 \mu\text{g} \mu\text{l}^{-1}$  yeast tRNA was added to the TE prior to bead-beating to enhance DNA recovery.

*Loma salmonae* rDNA was amplified using PCR, with primers described by Vossbrinck et al. (1987). The forward primer 530f (5'-GTGCCAGC(C/A)GCCGCGG-3') and reverse primer 580r (5'-GGTCCGTGTTCAA-GACGG-3') are located in the SSU and LSU rDNA genes, respectively (Fig. 1), and have been used successfully in several other microsporidia to amplify rDNA fragments of ca 1350 to 1550 bp in length (Vossbrinck et al. 1993, Zhu et al. 1994).

Each 50  $\mu\text{l}$  reaction used standard PCR buffer (Gibco BRL, Gaithersburg, MD, USA), 1.5 mM  $\text{MgCl}_2$ , and 0.2 mM dNTP, 25 pmol of each primer, 1.25 units of Taq DNA polymerase (Bethesda Research Laboratory, MD, USA), and 0.6  $\mu\text{g}$  of template DNA. The reactions were run in a Perkin Elmer GeneAmp 9600 Thermocycler (Perkin Elmer Cetus, Norwalk, CT, USA) for 30 cycles consisting of denaturation at  $94^{\circ}\text{C}$  for 1 min, primer annealing at  $50^{\circ}\text{C}$  for 1 min, and extension at  $72^{\circ}\text{C}$  for 3 min, which were preceded by an initial 3 min denaturation at  $95^{\circ}\text{C}$  and followed by a final 10 min  $72^{\circ}\text{C}$  extension. Negative controls, which used distilled water instead of template DNA, were included

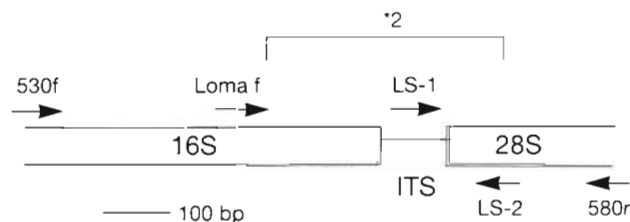


Fig. 1. Structural organization of the *Loma salmonae* rDNA fragment amplified with primers 530f and 580r; an additional 400 to 500 bp of the small subunit gene and 2500 to 3500 bp of large subunit are located at the 5'- and 3'-ends, respectively. Locations of primers Loma f, LS-1, and LS-2 are given, and the region sequenced in a second isolate of *L. salmonae* is indicated by the square bracket (\*2)

Fig. 2. Partial sequence alignment of *Loma salmonae* from chinook salmon and *Microsporidium* sp. from whitefish, demonstrating sequence variability in the regions of the *L. salmonae*-specific primers, LS-1 and LS-2. Dashes indicate gaps inserted in the sequence alignment, and asterisks show identical bases between the 2 species. Presumptive internal transcribed spacer region is shown in lower case letters, and numbers on the right indicate the number of nucleotides from the 3'-end of the 530f primer

		LS - 1		
		-----		
<i>Loma salmonae</i>	-	GTAGGAGAACCTGTAGCTG	atcagaccgattta-----	919
<i>Microsporidium</i> sp.	-	GTAGGAGAACCTGTAGCTG	atcagactgagagaaacgaaggag	939
---->				
<i>Loma salmonae</i>	-	tataatctttgtatgaatgtaat	CTCTGCGCAAGGGATCTT...	960
<i>Microsporidium</i> sp.	-	taaagttagaagagtaaaagaaaa	CCCTGCGCAAGGGATCTT...	980
<-----				
<i>Loma salmonae</i>	-	...GGA	ACTGCCCCGAGTGTAAATCA-----CAATTATGTGAGA	1167
<i>Microsporidium</i> sp.	-	...GG	AGGAGCCCAAAGTGTAAATTCATGAGCGGATCTGTTGAGA	1192
LS-2				
-----				
<i>Loma salmonae</i>	-	TGTCATTAATCTATAG---	AACGATGTTGRAACATCGGCCTGA	1207
<i>Microsporidium</i> sp.	-	TGTC	AAAAAAGCACGGCGTAACCAGTCTGGAACGGAGGCCTAA	1236

in each series of reactions to screen for possible contamination. The *Loma salmonae*-specific PCR product was excised from low melting point agarose and purified, according to the freeze-thaw method of Qian & Wilkinson (1991), for subsequent cloning and/or sequencing.

**Cloning and sequencing:** Primers 530f and 580r were synthesized to include EcoRI and BamHI restriction sites, respectively, at their 5'-ends. The PCR product from isolated *Loma salmonae* spores was cut with these restriction enzymes and cloned into the phagemid vector pBluescript II (SK- and SK+) (Stratagene Corp., La Jolla, CA, USA) by standard procedure (Sambrook et al. 1989). DNA was purified from overnight cultures by standard alkaline lysis miniprep protocol (Sambrook et al. 1989).

One clone within each of the SK- and SK+ vectors was selected, and the presence of the desired inserts were verified by restriction enzyme digests. Single-stranded DNA was generated from both clones using VCS-M13 helper phage (Stratagene). Both strands of the 2 clones were sequenced from single- and double-stranded DNA using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH, USA).

To examine variation in rDNA sequence between *Loma salmonae* isolates, the *L. salmonae*-specific PCR product obtained from the infected gills was reamplified with internal primers to yield ca 50 ng  $\mu\text{l}^{-1}$ . The PCR fragments were sequenced directly with the Sequenase PCR Product Sequencing Kit (USB) to generate 638 bp of sequence spanning the ITS region (Fig. 1).

**Development of the PCR test. Specimen collection:** For the development and initial testing of the *Loma salmonae* assay, chinook salmon tissues were collected from the above-mentioned seawater netpen farm as

follows. In March 1995, gills and kidneys from 14 moribund fish (avg. wt. ca 2 kg) were frozen at  $-70^{\circ}\text{C}$ . In addition, 3 control fish from Rosewall Creek were transported to the farm on ice and were sampled in between sampling of the suspect fish to evaluate the possibility of cross-contamination during the sampling process. In addition, 5 more control fish from the Rosewall Creek Hatchery were assayed at our laboratory. Tissue samples were also placed in Davidson's solution for histological analysis, as described previously.

**Oligonucleotide design and PCR conditions:** Presumptive *Loma salmonae*-specific PCR primers were designed by comparing the rDNA sequence generated above, particularly the hypervariable ITS region and variable portions of the 28S gene, with those available for other microsporidians found infecting fishes in British Columbia. The rDNA sequence of the only other microsporidian known to infect chinook salmon, *Nucleospora salmonis* (cf. Docker et al. 1997), showed less than 60% sequence homology with that of *L. salmonae* and the ITS regions showed no resemblance. Ribosomal DNA sequence from an undescribed *Microsporidium* sp. that we recently found in mountain whitefish *Prosopium willamsoni* muscle was ca 75% homologous with that of *L. salmonae*. *L. salmonae*-specific primers were designed from the regions that differed most between these species (Fig. 2). These primers, LS-1 and LS-2, amplify a 272 bp fragment.

The other PCR components were as described above, but the thermal profile was as follows: 35 cycles of  $94^{\circ}\text{C}$  for 1 min,  $53^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 1 min, preceded and followed by a 3 min denaturation and 10 min extension, respectively. All DNA extractions and PCR preparations were made with aerosol-resistant pipette tips, as contamination was found to be high otherwise.

**Sensitivity and specificity of the PCR:** To test the specificity of the LS-1 and LS-2 primers, they were used on DNA from *Nucleospora salmonis* from chinook, *Microsporidium* sp. from whitefish, and a closely related species, *Loma embiotocia*, which was recently reported in shiner perch *Cymatogaster aggregata* (cf. Shaw et al. 1997). To determine the level of sensitivity of the PCR test, DNA from gill tissue containing a known concentration of *L. salmonae* was extracted and serially diluted with DNA from a control fish until the desired PCR fragment was no longer detected; total DNA per 50  $\mu$ l reaction was constant at 0.6  $\mu$ g.

**Test refinements:** In an effort to streamline the PCR test and increase its sensitivity and reliability, the following aspects of the assay were examined. (1) The bead-beating method of DNA extraction was compared to proteinase K digestion and mechanical homogenization. In the former method, the tissues were digested in 1.5 ml eppendorf tubes at 37°C for 6 h to overnight (10 mM Tris, pH 8.0; 10 mM EDTA; 1% SDS; 150 mM NaCl; 200  $\mu$ g ml<sup>-1</sup> proteinase K) before phenol chloroform extraction and precipitation. In the latter, the tissue was homogenized in TE with mortar and pestle prior to extraction and precipitation. (2) The quantity of tissue sampled (30, 100, 200, or 300 mg) and the volume of the PCR (25, 50, or 100  $\mu$ l) were evaluated with regards to efficiency and repeatability. (3) DNA extraction and PCR from tissues preserved in ethanol or previously embedded in paraffin (following recovery through a series of xylene and alcohol) were compared to results from frozen tissues.

**Broodstock screening. Specimen collection:** Given the observation of *Loma salmonae* DNA in the ovary of a chinook salmon (see 'Results'), the *L. salmonae* PCR assay was used to screen various tissues from females used for broodstock at the above-mentioned fish farm. Tissues were collected as follows: In July and August 1995, gills, kidneys, and ovaries from 26 spawned females were frozen, and the DNA from two 100 mg subsamples was extracted from each by proteinase K digestion. In the ovarian samples, DNA was extracted from connective tissue and/or eggs, depending on availability, and where possible, tissues were also preserved for histological analysis (see Table 2). These females hatched and reared in fresh water at a hatchery in mainland British Columbia and then in seawater netpens on Vancouver Island. The ovarian tissue of an additional 48 females from another population at this fish farm were examined with the PCR test only. In addition to PCR with primers LS-1 and LS-2, a general *Loma* sp. primer (*Loma* f, 5'-ATTAGTGAGACCTCAGCC-3') was also paired with LS-2, in case geographic differences in ITS sequence prevented LS-1 from annealing; *Loma* f and LS-2 amplify a 627 bp fragment in *L. salmonae*. During the extraction procedure, Atlantic salmon liver was also processed to

screen for possible contamination prior to PCR. Many samples from the broodstock study were negative for *L. salmonae* by PCR analysis. Therefore, to determine whether amplifiable template was present, 47 of these samples were tested with control primers developed from conserved salmonid growth hormone (GH) gene sequences (Devlin unpubl.).

**PCR conditions:** PCR components and conditions were as described above in the development of the PCR test, and reactions were performed in duplicate.

## RESULTS

### *Loma salmonae* ribosomal DNA sequence

The PCR primers 530f and 580r produced a single 1450 bp fragment with DNA from purified *Loma salmonae* spores. This PCR product was also evident with DNA from infected gill tissue, although several smaller bands were also present. These other bands, which were also present in the uninfected chinook samples, have been shown to be derived from salmon DNA (Docker et al. 1997).

Upon sequencing, the amplified fragment was found to consist of 1391 bp of *Loma salmonae* rDNA: ca 905 bp of SSU rDNA, 37 bp of ITS, and 449 bp of the LSU gene, although the exact boundaries of the ITS are not known. Two nucleotide differences were noted between the 2 cloned sequences (at positions 574 and 1329), and only 1 difference was found in the 638 bp region sequenced in both isolates (position 1194); all differences were transitions. The sequence has been deposited in GenBank (accession no. U78736).

### Development of the PCR test

Oligonucleotide primers were designed from species-specific regions of the *Loma salmonae* rDNA sequence (Fig. 2), avoiding the sites of polymorphism mentioned above. *Loma salmonae* rDNA was detected in gill and/or kidney tissue in several fish from the seawater netpen farm (Table 1). Two of these fish exhibited no histological signs of *L. salmonae* infections, while others had xenomas or chronic, multifocal branchitis identical to that previously associated with *L. salmonae* infections (Kent et al. 1989, 1995, Speare et al. 1989). In some fish with bacterial kidney disease or plasmacytoid leukemia, *L. salmonae* infections were identified by PCR but not by histology (Table 1). Duplicate PCR samples concurred and there was no amplification in the negative controls, including those collected at the farm site, suggesting that the positive results were not due to contamination.

Table 1. *Loma salmonae* in seawater pen-reared chinook salmon. PCR results compared to histological analysis. -: negative; +: positive; ++: strong positive; CMB: chronic, multifocal branchitis; RC: Rosewall Creek control stock; BKD: bacterial kidney disease; PL: plasmacytoid leukemia with *Nucleospora salmonis* infection (unless otherwise indicated)

Fish no.	PCR		Histology
	Kidney	Gill	
1-5	-	-	Normal—Controls from RC
6	-	-	BKD
7	-	-	Normal—RC control sampled at farm
8	+	-	PL
9	-	-	Normal—RC control sampled at farm
10	+	-	<i>L. salmonae</i> xenomas in gills
11	-	+	BKD with CMB
12	-	-	Normal—RC control sampled at farm
13	-	-	Normal
14	-	-	BKD and PL with no <i>N. salmonis</i>
15	-	-	BKD with CMB
16	-	-	CMB
17	++	++	<i>L. salmonae</i> xenomas in gills
18	-	++	PL with CMB. One <i>L. salmonae</i> spore observed in 1 granuloma
19	-	+	BKD, with 1 small focus of inflammation in gill

Of 4 microsporidians assayed in the specificity test, the LS-1 and LS-2 primers amplified DNA only from *Loma salmonae* (Fig. 3). In the sensitivity test, DNA originating from as few as 0.01 spores could routinely be detected in a 50  $\mu$ l PCR (Fig. 3) and a faint PCR product was sometimes visible at 0.001 spores per reaction. Consequently, under the conditions described, the PCR assay is capable of detecting between 4 and 40 spores  $g^{-1}$  of gill tissue. PCR with the more general primer pair, Loma f and LS-2, was capable of detecting between 1 and 10 spores per reaction.

Evaluation of the various aspects of the assay led to the following observations: (1) Of the 3 methods of DNA extraction compared, all were equivalent in terms of the recovery of amplifiable *Loma salmonae* DNA. In terms of ease of handling, however, the proteinase K method was least labour-intensive. Mechanical homogenization was moderate in complexity, requiring multiple homogenizers or extremely thorough cleaning between each sample. The bead-beating method involved the most handling and was more prone to cross-contamination than the others. A 6 h proteinase K digestion was found to be sufficient, as was a single phenol chloroform extraction. (2) DNA from 30 and 100 mg of tissue could easily be extracted in 1.5 ml eppendorf tubes,

but larger quantities required the use of tubes which would no longer fit in the benchtop centrifuge and limited the numbers of tests that could be performed. Of 25, 50, and 100  $\mu$ l, PCR volumes of 50  $\mu$ l were preferred: all were found to be equivalent at high levels of infection, but light infections could only sporadically be detected at 25  $\mu$ l and any slight increase in the sensitivity of the assay at 100  $\mu$ l was not considered to be worth the added expense. (3) DNA could easily be amplified by PCR from tissues which had been frozen or preserved in ethanol, but not from formalin-fixed paraffin-embedded tissues, using these extraction methods.

### Broodstock screening

Of 26 females examined, 2 fish were found to have light or sporadic infections in the gills based on PCR results.

In these fish, *Loma salmonae* spores were not detected by histology, i.e. either the gills were normal or they exhibited multifocal, chronic branchitis with no evidence of spores within the lesions (Table 2). The kidney of 1 fish was positive by the PCR test, and was negative by histology. In only 1 fish did the PCR fail to detect an infection which had been observed by histology. *L. salmonae* rDNA was found in the ovaries of 4 females by PCR, and the presence of xenomas in

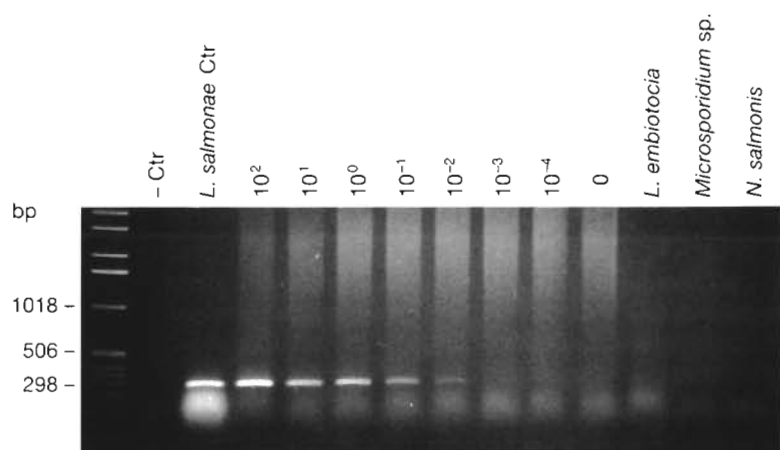


Fig. 3. Sensitivity and specificity of the *Loma salmonae* primers LS-1 and LS-2 (1% agarose gel, ethidium-bromide stained). The negative and positive controls (Ctr), respectively, used distilled water and DNA from purified *L. salmonae* spores. DNA for the dilution study was extracted from gill tissue containing a known number of spores per gram. Molecular weight markers (base pairs) are shown in the left lane

Table 2. Detection of *Loma salmonae* in various tissues of mature female chinook salmon. Where available, results obtained by PCR and histology are compared. Ovary refers to ovarian connective tissue; -: negative; +: positive; ++: strong positive; blank space indicates tissue not tested; CMB: chronic multifocal branchitis

Fish no.	Gill		Kidney		Ovary		Egg	
	PCR	Histology	PCR	Histology	PCR	Histology	PCR	Histology
1	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-
20	-	CMB	-	-	-	-	-	-
21	-	CMB	-	-	-	-	-	-
22	+ <sup>d</sup>	-	+ <sup>d</sup>	-	-	-	-	-
23	-	-	-	-	+	-	-	-
24	-	-	-	-	++	-	-	-
25	+ <sup>d</sup>	CMB	-	-	++	++	-	-
26	-	++	-	-	++	++	-	-

<sup>d</sup>One sample consistently tested + and the other -

ovarian blood vessels was verified by histology in 2 of these fish (Fig. 4). *L. salmonae* was not detected in the eggs themselves, either by PCR or by histology, including 3 other females in which the ovarian tissue tested positive for the parasite (Table 2). Because of the occurrence of *L. salmonae* in the ovaries, this organ was examined by PCR from an additional 48 broodstock females. *L. salmonae* was detected in the ovaries from 22 of these fish by this method. To confirm that the ca 270 bp fragment amplified from field samples was *L. salmonae* rDNA, we sequenced the PCR product from 10 of these positive ovary samples. The sequence from all samples concurred with *L. salmonae* rDNA. Of the 47 negative samples tested with conserved GH primers, 46 were found to contain amplifiable template, demonstrating that these samples were not negative due to DNA extraction problems.

## DISCUSSION

The PCR test described in this study was capable of detecting very small amounts of *Loma salmonae* DNA, even when mixed with a very large amount of host

chinook salmon DNA. The specificity and sensitivity of this test make it a valuable tool for the study of *L. salmonae* transmission (e.g. identification of sources of infection), detecting early or very mild infections, and for routine broodstock screening

The *Loma salmonae*-specific primers LS-1 and LS-2 failed to amplify DNA from the other microsporidia tested. The specificity of these primers allows for the differentiation of closely related, and possibly morphologically indistinguishable, species. For example, using these primers and a more conserved primer set, Shaw et al. (1997) substantiated that a *Loma* species from shiner perch *Cymatogaster aggregata* was a different species than *L. salmonae*, and it was thus given the name *L. embiotocia*. This was an important observation because shiner perch are common around seawater netpens in British Columbia and were considered a potential reservoir host for *L. salmonae*. The LS-1 and LS-2 primers will also amplify *L. salmonae* from other geographic regions. For example, these primers are capable of amplifying rDNA of *L. salmonae* isolates from California and

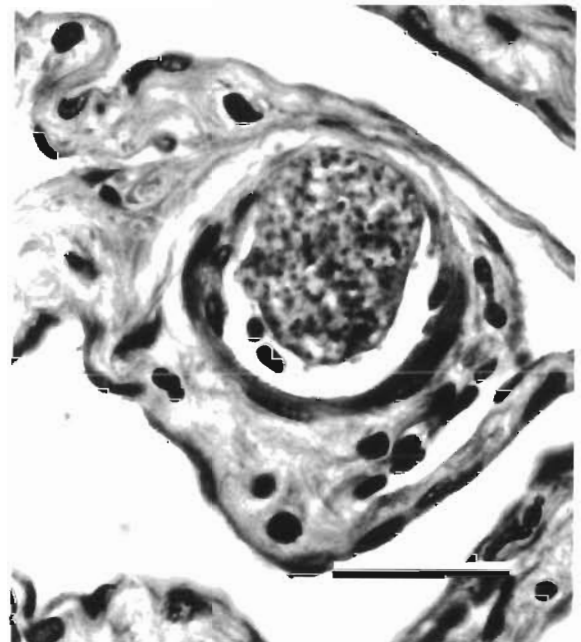


Fig. 4. *Oncorhynchus tshawytscha*. Blood vessel in the ovary with *Loma salmonae* xenoma. Hematoxylin & Eosin. Scale bar = 25  $\mu$ m

Idaho, USA (A. Brown, Department of Zoology, University of British Columbia, Vancouver, B.C., pers. comm.).

The *Loma*-positive PCR reaction in fish with plasmacytoid leukemia and *Nucleospora salmonis* infections (Table 1) was most likely due to a light infection by *L. salmonae* because the test was specifically shown not to react with the former microsporidium. Nevertheless, in situations where other closely related microsporidia are suspected, the LS-1 and LS-2 primers should be tested with other microsporidian species to broaden the conclusion that they are species specific. Barlough et al. (1995) reported a nested PCR assay for *Enterocytozoon (Nucleospora) salmonis* which also tested positive for a microsporidium that infects insects, *Nosema necatrix*. Such cross-reactivity, however, is of less concern than that from organisms which may also exist in salmon.

In addition to its specificity, the PCR assay for *Loma salmonae* offers a high degree of sensitivity. Both the logarithmic amplification of the PCR and the gene dosage per cell of ribosomal DNA confer a high degree of sensitivity on the assay. Although we do not know how many copies of rDNA are present in each *L. salmonae* spore, our ability to detect between 0.01 and 0.001 spores per reaction suggests at least several hundred. It appears, however, that the factor that may limit the assay's sensitivity is the amount of tissue from which the DNA is extracted. Because the PCR was capable of detecting as few as 4 to 40 spores g<sup>-1</sup> of gill tissue, light infections may be missed in sampling only 100 mg of tissue, especially if the spore distribution is patchy. This is the case with *L. salmonae*, in which spores are concentrated within discrete xenomas. DNA extraction from larger amounts of tissue was not practicable in 1.5 ml eppendorf tubes, but replicate 100 mg samples can be used where it is important to detect very light or localized infections. The small fragment size amplified by primers LS-1 and LS-2 (272 bp) also improved the sensitivity of the assay by increasing the amplification efficiency; PCR using primers that amplify a 627 bp fragment of *L. salmonae* rDNA were 3 orders of magnitude less sensitive.

Multifocal, chronic branchitis is a common lesion found in salmon in which xenomas have ruptured (Kent et al. 1989). However, spores are often difficult to detect in these lesions, and this lesion may be observed with other diseases, such as bacterial kidney disease. Our PCR test, therefore, can be useful for elucidating the cause of these lesions. For example in 1 fish in our study, only 1 spore was observed by histology, while the PCR test gave a strong reaction (Table 1).

The demonstration of *Loma salmonae* in ovarian tissue was largely the result of the PCR assay's sensitivity. Detection of *L. salmonae* rDNA in the ovary of 1 fish during the test's development prompted the

ovarian tissue of subsequent broodstock females to be histologically examined with great care, whereupon xenomas were clearly observed in the blood vessels.

*Loma salmonae* infections have been observed in other vascularized tissues such as the heart, spleen, kidney, and pseudobranch (Kent et al. 1989, Markey et al. 1994), but have not previously been reported in the ovary. Although the presence of the parasite in the ovary alone does not imply true vertical transmission, it is of concern for fish farmers using *L. salmonae*-infected broodstock. *L. salmonae* was not detected in the eggs themselves, but it is possible that the progeny of infected females may become exposed to the parasite through contaminated ovarian fluid. In 2 other microsporidians of fish, *Pleistophora (Plistophora) mirandellae* and *P. ovariae*, spores were found within mature ova of the hosts (Vaney & Conte 1901, Summerfelt & Warner 1970, respectively), but there was no direct evidence of transovarian infection because the infected eggs were fragile and ruptured easily (Summerfelt & Warner 1970). Nevertheless, these infected eggs appeared to release spores that subsequently attached to previously uninfected eggs, and atresia of infected ova may also release numerous spores into the ovary (Summerfelt & Warner 1970). Progeny of control and infected female chinook salmon are currently being reared in *L. salmonae*-free well water to determine if the latter group will develop infections. If such is the case, routine screening of broodstock females with the PCR assay described in this study may control the spread of *L. salmonae* to subsequent generations.

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