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Genetic characterization and experimental pathogenesis of *Piscirickettsia salmonis* isolated from white seabass *Atractoscion nobilis*

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ABSTRACT: An intracellular bacterium originally isolated from hatchery-reared juvenile white seabass *Atractoscion nobilis* in southern California, USA, was identified by sequences of the small and large subunit ribosomal (16S and 23S) DNA and the internal transcribed spacer (ITS) as *Piscirickettsia salmonis*. Considering all rDNA sequences compared, the white seabass isolate (WSB-98) had a 96.3 to 98.7% homology with 4 previously described strains of *P. salmonis* isolated from salmon in Chile, Norway, and British Columbia, Canada. Experimental infections induced by intraperitoneal injections of juvenile white seabass with WSB-98 resulted in disease and mortality similar to that observed in *P. salmonis* infections in salmon. After 60 d, the cumulative mortality among *P. salmonis*-injected white seabass was 82 and 40%, respectively, following a high (1.99×10^4 $TCID_{50}$) or low (3.98×10^2 $TCID_{50}$) dose-challenge with WSB-98. The bacterium was recovered by isolation in cell culture or was observed in stains from tissues of injected white seabass but not from control fish. There were no external signs of infection. Internally, the most common gross lesion was a mottled appearance of the liver, sometimes with distinct nodules. Microscopic lesions were evident in both the capsule and parenchyma of the liver and were characterized by multifocal necrosis, often with infiltration of mononuclear leukocytes. Macrophages filled with bacteria were present at tissue sites exhibiting focal necrosis. Foreign body-type granulomas were prevalent in livers of experimentally infected white seabass, but not in control fish. Similar granulomatous lesions were observed in the spleen, kidney, intestine and gills, but these organs were considered secondary sites of infection, with significantly fewer and less severe histologic lesions compared to the liver. The results from this study clearly indicate that infections with *P. salmonis* are not restricted to salmonid fishes and that the bacterium can cause a disease similar to piscirickettsiosis in nonsalmonid hosts.

KEY WORDS: Rickettsia-like · Seabass · *Piscirickettsia salmonis* · 16S rRNA

INTRODUCTION

Piscirickettsia salmonis is a small Gram-negative intracellular bacteria currently classified with the gamma subdivision of proteobacteria in the new family Piscirickettsiaceae (Fryer & Lannan in press). *Piscirickettsia* is 1 of 5 genera in the family and currently contains only 1 species, *P. salmonis* (Fryer & Hedrick 2003). The bacterium is associated with piscirickettsiosis, a significant disease of salmon reared in seawater net pens in Chile, Norway, Scotland, Ireland, and the Atlantic and Pacific coasts of Canada (Cvitanich et al. 1991, Brocklebank et al. 1992, Evelyn 1992, Fryer et al. 1992, Rodger & Drinan 1993, Cusak et

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al. 1997, Olsen et al. 1997). Coho salmon *Oncorhynchus kisutch* were originally the principal hosts when *P. salmonis* was first detected in Chile (Garcés et al. 1991). The presence of the bacterium and piscirickettsiosis now have been detected in rainbow trout *O. mykiss*, Chinook salmon *O. tshawytscha*, Atlantic salmon *Salmo salar*, pink salmon *O. gorbuscha*, and masou salmon *O. masou* (Garcés et al. 1991, Bravo 1994). Similar bacteria, referred to as *P. salmonis*-like organisms, have been observed in white seabass *Atractoscion nobilis* (M. F. Chen et al. 2000), black seabass *Dicentrarchus* sp. (Comps et al. 1996, Steiropoulos et al. 2002), grouper *Epinephelus melanostigma* (S. C. Chen et al. 2000), three-line grunt *Parapristipoma trilineatum* (Fukuda et al. 2002), tilapia *Oreochromis*, *Tilapia*, *Sarotherodon* spp. (Chen et al. 1994, Chern & Chao 1994), and blue-eyed plecostomus *Panaque suttoni* (Khoo et al. 1995).

Comparisons of the 16S and 23S ribosomal RNA genes and the internal transcribed spacer (ITS) regions of several geographically distinct isolates of *Piscirickettsia salmonis* show they cluster closely into a monophyletic group (Mauel et al. 1999, Heath et al. 2000). Sequence homologies among isolates of *P. salmonis* from salmon for these ribosomal genes ranged from 95.2 to 99.7% (Mauel et al. 1999). Experimental transmission studies with isolates from Chile (LF-89), Norway (NOR-92) and Canada (ATL-4-91) used in the genetic comparisons by Mauel et al. (1999) demonstrated that all were pathogenic to coho salmon, with LF-89 significantly more virulent than the NOR-92 isolate (House et al. 1999).

Piscirickettsiosis generally begins 4 to 5 wk following the introduction of salmon or rainbow trout to seawater net pens (Bravo & Campos 1989, Cvitanich et al. 1991). Infections in fresh water have been reported but are rare (Bravo 1994, Gaggero et al. 1995). A marine reservoir for *Piscirickettsia salmonis* has been suggested, since salmon are not native to the southern hemisphere (Donaldson & Joyner 1983) but are delivered to Chile principally as eggs, which are viewed as an unlikely source of the bacterium (Fryer & Hedrick 2003). In addition, the bacterium rapidly loses viability in fresh water (Lannan & Fryer 1994), where egg incubation and early salmon rearing occur. Rickettsia-like and *P. salmonis*-like organisms have been detected from a variety of marine fish species, including juvenile white seabass in southern California, USA (M. F. Chen et al. 2000). The infected white seabass from California were from a private hatchery that has raised fish since 1995 as part of a larger effort to restore this popular sport and commercial fishery (Vojkovich & Reed 1983). The disease observed in white seabass had similar gross and microscopic pathological features to piscirickettsiosis in salmon, but infections were more prominent in the liver compared to other organs examined (M. F. Chen et al. 2000). The bacterium (WSB-98) as isolated from white seabass in cell culture (CHSE-214) reacted weakly with anti-*P. salmonis* rabbit serum, the confirmatory test for *P. salmonis* (Office International des Epizooties 2000). Despite this weak reaction with anti-*P. salmonis* serum, WSB-98 induced a disease identical to piscirickettsiosis in juvenile coho salmon, resulting in an 80% cumulative mortality within 10 d following intraperitoneal injections of the bacterium (M. F. Chen et al. 2000).

In this report, we provide the first genetic characteristics of the bacterium from white seabass as compared to known strains of *Piscirickettsia salmonis* from salmon. In addition, we describe details of the sequential microscopic pathology of the disease following intraperitoneal injections of the bacterium into juvenile white seabass.

MATERIALS AND METHODS

Genetic comparisons. The rDNA sequences of *Piscirickettsia salmonis* isolates were amplified using the primers described by Mauel et al. (1999). The PCR reactions were carried out in 1× buffer containing 10 mM Tris-HCl pH 8.3 (at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% w/v gelatin, 400 µM dNTPs, 5 µM tetramethyl ammonium chloride, 40 pmol of each primer, and 2 U *Taq* DNA polymerase (Invitrogen). Amplification was performed as described by Mauel et al. (1999) using a PTC-100 thermal cycler (MJ Research). After amplification, products were evaluated by ethidium bromide agarose gel electrophoresis. PCR testing of the white seabass bacterium was performed using the type-strain-specific primers described by Mauel et al. (1996).

Amplicons of the *Piscirickettsia salmonis* rDNA were purified using PCR spin-columns (Qiagen) and submitted to a commercial laboratory for sequencing (Davis Sequencing LLC). Both forward- and reverse-strand sequences were obtained using the same original primers as those used in the PCR. Sequences were aligned and compared using Mac DNAsis v3.7 (MiraiBio). Sequences obtained for 816 bp of the 16S, 203 bp of the ITS and 309 bp of the 23S rDNA were compared to those of 4 isolates of *P. salmonis* obtained from salmon in Chile (LF-89, [GenBank Accession Nos. U36941, U36943] and EM-90 [Accession Nos. U36940, U36944]), Norway (NOR-92; Accession Nos. U36942, U36946) and British Columbia, Canada (ATL-4-91; Accession Nos. U36915, U36945).

Fish. Juvenile white seabass *Atractoscion nobilis* used in this study originated from the Leon Raymond Hubbard Jr Marine Fish Hatchery, a Hubbs-SeaWorld

Research Institute facility supplied with seawater from a local lagoon in Carlsbad. The fish were transported to the pathogen-containment facility at the Bodega Marine Laboratory, Bodega Bay, California. The white seabass were held in fiberglass tanks supplied with flow-through seawater maintained at 15 to 16°C, and were fed a commercial ration at 2% body weight (BW) d^{-1}. At the beginning of exposure, the fish were 128 d old.

Growth of bacterium. The bacterium WSB-98 from white seabass, originally isolated from liver tissue of moribund white seabass (M. F. Chen et al. 2000), was propagated in the salmonid cell line CHSE-214 (Lannan et al. 1984) at 20°C as previously described (M. F. Chen et al. 2000). Bacterial concentrations were estimated by 50% tissue culture infective dose ($TCID_{50}$) assays on CHSE-214 cells as described by Fryer et al. (1990), except that the cells were incubated at 20°C for 21 d. Calculations for the concentrations of the WSB-98 used endpoint dilutions and the method of Reed & Muench (1938).

Virulence for white seabass. The virulence of WSB-98 was evaluated in juvenile white seabass (mean BW 23 g, SD 8 g) by intraperitoneal (i.p.) injection. A total of 300 fish were used in the experiment. Immediately prior to exposure, 50 fish were placed into each of 6 replicate 215 l tanks supplied with 15 to 16°C aerated seawater with a flow rate of 0.2 l min^{-1}. For each of 2 replicate tanks (A and B), fish were injected intraperitoneally with either 1.99×10^4 $TCID_{50}$ WSB-98 ('high dose') or 3.98×10^2 $TCID_{50}$ WSB-98 ('low dose') per fish with the culture medium from CHSE-214 cells infected 7 d earlier with the bacterium; 2 replicate tanks of 50 fish each received only culture medium from uninfected CHSE-214 cells. At selected time points, up to 4 fish were removed from 1 replicate tank at the high, low, and control doses and subjected to standard necropsy procedures including impression smears of the liver, collection of portions of the liver, kidney, spleen, posterior intestine and gill for histopathological examination, and in some cases reisolation of the bacterium from the kidney using CHSE-214 cells. Fish in the remaining replicate tanks, for each of the 3 treatments (high and low dose, control), were not sequentially sampled, but instead were used to determine the cumulative mortality over the study period. Dead or severely moribund fish were removed from all tanks and impression smears prepared to detect presence of the bacterium. Dead or moribund fish were not included among fish sampled for the study of sequential development of the disease. From the high-dose exposures, fish were sampled 1, 3, 7, 10, 14, 21, 30, and 60 d post-inoculation. From the low-dose exposures, fish were sampled 7, 14, 21, 28, 35, 42, 49, and 60 d post-inoculation. Fish were collected from the control group on all sampling dates. Tissues for histological evaluation were placed in Davidson's solution (Humason 1979) for approximately 16 h and then transferred to 70% ethanol. Tissues were then processed for routine paraffin-embedding, sectioning and staining with hematoxylin and eosin (H&E). Imprints made directly from liver tissues were air-dried for 1 h, fixed in 100% methanol for 5 min, and then stained with May-Grünwald Giemsa.

Reisolation of WSB-98. Reisolation and determination of the concentrations of WSB-98 in the kidney, spleen, and/or liver of injected white seabass were used to evaluate infection intensity during the peak of mortality post-injection. Tissues from freshly dead fish were removed aseptically and homogenized in minimal essential medium without sodium bicarbonate, supplemented with 2% fetal bovine serum, 20 mM L-glutamine and buffered with 25 mM HEPES (MEM-2+HEPES). The homogenate was centrifuged at $208 \times g$ at 4°C for 1 min and the resulting supernatant was serially diluted in MEM-2+HEPES. Concentrations of the bacterium in fish tissues were estimated by $TCID_{50}$ assays on CHSE-214 cells.

Fluorescent antibody tests. Fluorescent antibody tests with anti-WSB-98 rabbit serum were conducted on methanol-fixed impression smears from the liver of fish collected during the study. The rabbit serum was prepared as previously described by M. F. Chen et al. (2000) and was used in indirect fluorescent antibody tests (IFAT) as described for *Piscirickettsia salmonis* by Lannan et al. (1991), with some modifications. Additionally, immunofluorescence with the anti-WSB-98 serum was used to confirm that isolates obtained from tissue homogenates of fish in the experimental challenges were WSB-98. Briefly, supernatants and CHSE-214 cells collected from culture plate wells were centrifuged at $16\,000 \times g$. The resulting pellet was resuspended in 100 µl phosphate-buffered saline (PBS), and 20 µl of this sample was spotted onto a slide, air-dried for 1 h and then fixed in 100% methanol for 5 min. Both impression-smear and supernatant samples were blocked with a solution of 10% nonfat milk and 0.05% Tween in PBS (PBS-T) for 30 min and then incubated with rabbit anti-WSB-98 serum diluted 1:500 in 1.5% nonfat milk-PBS-T for 1 h. Slides were rinsed with PBS-T and then incubated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Kirkegaard-Perry) diluted 1:40 in 1.5% nonfat milk-PBS-T. After 1 h, the slides were rinsed as before, counterstained with 0.01% Evan's blue in PBS, rinsed with PBS and mounted with buffered glycerol for microscopic evaluation.

Histological evaluation. Liver, spleen, gill, kidney and posterior intestine from 109 juvenile white seabass were assessed for histologic lesions. Slides with H&E-stained tissue sections were randomly mixed and read

blind (i.e. without knowledge of exposure history, sampling dates or group designations). Prevalence was determined for each lesion, and comparisons made between the 3 exposure groups (high, low, control). Prevalences were sorted a second time—on the basis of sampling date—to assess lesion development over time. The results were grouped into 3 time frames (0 to 1, 1 to 3 and 3 to 8 wk post-exposure).

RESULTS

Genetic comparisons

Using primer sets PS2S and PS3AS, an 816 bp amplicon of the 16S rDNA of the WSB-98 was obtained. A second PCR with primer sets PS16S and PS23S resulted in an amplicon of 536 bp which, upon sequencing, contained 22 bp from the 3′ end of the 16S rDNA, the total ITS region of 309 bp, and 203 bp from the 5′ end of the 23S rDNA. An alignment comparing the WSB-98 sequence to those from *Piscirickettsia salmonis* isolates from Chile LF-89 and EM-90, Norway NOR-92, and British Columbia Canada ATL-4-91 indicated that differences between isolates occurred at 64 loci (Table 1). A pairwise comparison between all rDNA sequences obtained (a total of 1328 bp) demonstrated that WSB-98 was most closely related to NOR-92 (98.7%), with lesser homologies to LF-89 (98.5%), ATL-4-91 (98.4%) and least of all to EM-90 (96.3%) (Table 2). Using the diagnostic PCR test described by Mauel et al. (1996), an amplicon of 467 bp was obtained which upon sequencing differed from LF-89 by 4 bp (data not shown).

Experimental infections

Over a 60 d post-exposure observation period, peak mortality in the high-dose group occurred between 1 and 3 wk, with cumulative mortality reaching 82% (41/50) (Fig. 1). In the low-dose group, peak mortality occurred approximately 2 wk later, from 3 to 5 wk, reaching a 40% (20/50) cumulative rate. A total of 13/50 (26%) of the white seabass in the control group died. The 13 dead control individuals were autolyzed

Table 1. Comparison of loci in regions of 16S, ITS, and 23S rDNA sequences differing among Isolate WSB-98 from white seabass *Atractoscion nobilis* in California and 4 isolates of *Piscirickettsia salmonis* from salmon in Chile (LF-89 type strain, EM-90), Norway (NOR-92) and Canada (ATL-4-91). Only those locations where differences were detected are shown. Base position numbering according to Mauel et al. (1999)

Base position:	269	374	387	389	392	426	519	537	612	677	715	725	874	876	877	891	894	895	910	911
P. salmonis LF-89 16S	G	G	G	A	G	G	C	T	C	G	G	G	A	C	G	G	G	C	A	A
WSB-98 16S	G	G	G	A	G	G	C	T	C	G	G	G	A	C	G	G	G	C	A	A
P. salmonis ATL-4-91 16S	G	G	G	A	G	G	C	T	C	G	G	–	C	–	–	C	C	C	A	–
P. salmonis NOR-92 16S	G	G	G	A	G	G	C	T	A	G	G	G	C	C	–	C	C	–	C	A
P. salmonis EM-90 16S	A	A	–	–	–	–	G	C	C	–	C	–	A	C	C	C	C	C	A	A

Base position:	920	924	929	930	931	936	958	963	1003	1006	1007	1008	1009	1010	1017	1018	1019	1022
P. salmonis LF-89 16S	C	G	C	A	–	–	T	–	–	C	T	–	–	C	G	C	G	A
WSB-98 16S	C	G	G	C	A	–	T	G	–	C	T	T	G	C	G	C	G	A
P. salmonis ATL-4-91 16S	G	C	C	A	–	–	T	G	–	C	T	–	–	C	G	C	G	A
P. salmonis NOR-92 16S	C	G	C	C	A	–	T	–	–	C	T	C	–	C	G	C	G	A
P. salmonis EM-90 16S	C	G	–	C	A	G	–	–	G	G	A	T	–	–	A	T	T	T

Base position:	61	66	140	143	145	151	163	164	167	169	177	179	181	183	193	248	260	275
P. salmonis LF-89 ITS	T	A	G	A	G	A	–	–	C	T	T	T	A	A	T	G	A	T
WSB-98 ITS	T	A	G	A	G	A	A	T	C	G	G	T	A	A	–	A	T	A
P. salmonis ATL-4-91 ITS	T	A	T	A	G	A	A	T	C	G	G	T	A	A	–	A	T	A
P. salmonis NOR-92 ITS	T	A	T	A	G	G	A	T	C	G	G	T	A	A	T	A	T	A
P. salmonis EM-90 ITS	A	C	T	C	A	A	–	–	T	T	A	A	G	G	T	G	T	T

Base position:	109	118	119	156	165	171	199
P. salmonis LF-89 23S	C	C	T	G	A	T	A
WSB-98 23S	G	C	T	G	–	G	C
P. salmonis ATL-4-91 23S	G	T	C	G	A	T	A
P. salmonis NOR-92 23S	G	C	T	G	A	G	A
P. salmonis EM-90 23S	G	C	T	A	A	T	A

Table 2. *Piscirickettsia salmonis*. Percent homologies of regions of 16S, ITS and 23S ribosomal DNA (rDNA) sequences of Isolate WSB-98 from white seabass *Atractoscion nobilis* compared to 4 isolates from salmon. Combined percent homology considers all differences in rDNA sequences observed

rDNA	Isolate from salmon			
	LF-89	ATL-4-91	NOR-92	EM-90
16S	99.0	98.2	98.5	96.6
ITS	96.1	99.5	98.5	92.1
23S	98.7	98.4	99.4	98.7
Combined	98.5	98.4	98.7	96.3

and the cause of death could not be determined. The bacterium was recovered from 5/6 dead WSB-98-exposed fish tested, in a range of 1.58×10^4 to 2.34×10^6 $TCID_{50}$ g^{-1} (1 culture was contaminated), and the IFAT confirmed that these isolates were WSB-98. Impression smears of liver tissues stained with Giemsa or subjected to IFAT were used to demonstrate the presence of *Piscirickettsia salmonis* in dead and sampled fish throughout the 60 d experiment (Table 3). In the high- or low-dose exposure groups, detection of the bacterium by either Giemsa or IFAT was similar across all sampling points. In the sampled groups, detection of WSB-98 was greatest in the 1 to 3 wk post-exposure period, with 10/12 (83.3%) and 5/8 (62.5%) positive, respectively, in the high- and low-dose exposure groups. In the replicate tanks, in which the disease was allowed to proceed and from which only moribund or dead individuals were removed, WSB-98 was detected in 97.6% (40/41) of the fish in the high-dose exposure group and 80% (16/20) of those in the low-dose group. No *P. salmonis* bacteria were detected in stained impression smears taken from dead or sampled fish from the control groups.

Table 3. *Piscirickettsia salmonis*. Detection in stained liver impression smears from white seabass *Atractoscion nobilis* following experimental exposures to Isolate WSB-98 in replicate tanks (see 'Materials and methods'). Values are no. positive/no. sampled. IFAT: indirect fluorescent antibody test

Time frame	High dose		Low dose		Control	
	Giemsa	IFAT	Giemsa	IFAT	Giemsa	IFAT
Tanks A						
Sampled 0–1 wk	0/12	1/12	0/4	0/4	0/12	0/12
Sampled 1–3 wk	10/12	10/12	5/8	5/8	0/12	0/12
Sampled 3–8 wk	2/5	2/5	4/18	3/18	0/17	0/17
Tanks B						
Mortality 0–1 wk	1/2	2/2	0/2	0/2	0/10	0/10
Mortality 1–3 wk	36/36	36/36	8/8	8/8	0/1	0/1
Mortality 3–8 wk	2/3	2/3	8/10	7/10	0/2	0/2
Sampled at 60 d	1/9	0/9	0/10	0/10	0/10	0/10

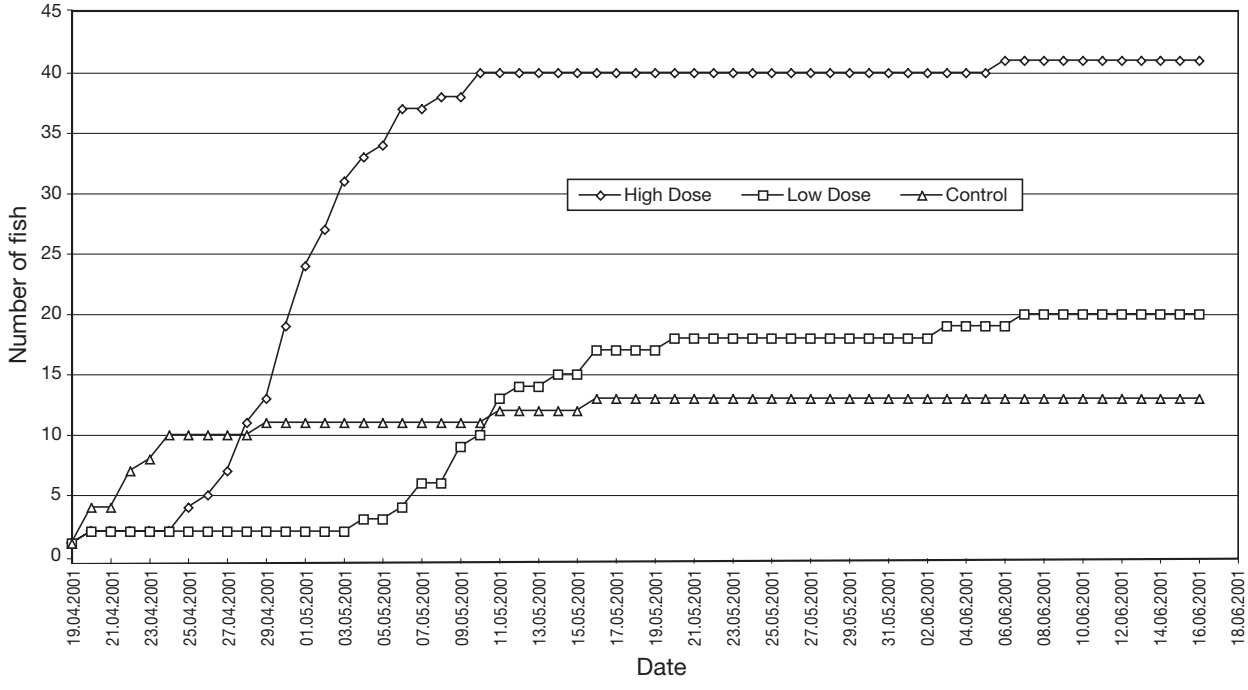

Fig. 1. *Atractoscion nobilis*. Cumulative mortality in white seabass following experimental exposure to *Piscirickettsia salmonis* WSB-98. Dates are d/mo/yr

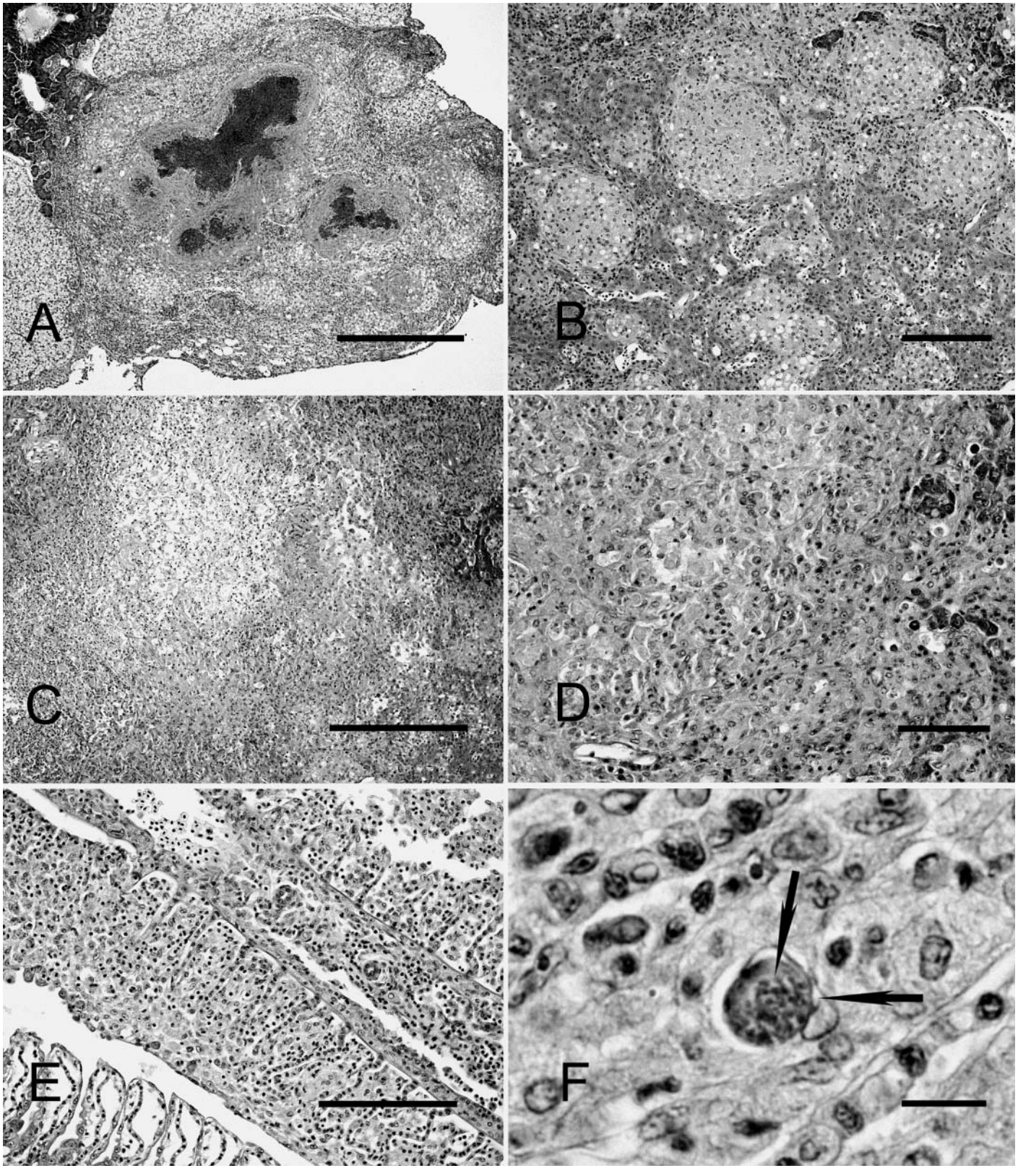

Fig. 2. *Atractoscion nobilis*. H&E sections of white seabass experimentally exposed to *Piscirickettsia salmonis* WSB-98. (A) Granulomas in liver; (B) macrophage aggregates in liver; (C) focal necrosis in liver; (D) focal necrosis in liver; (E) granulomatous inflammation in gill; (F) intracellular bacteria (arrows) in gill. Scale bars: (A) = 500 µm, (B) = 100 µm, (C) = 200 µm, (D) = 50 µm, (E) = 100 µm, (F) = 10 µm

Sequential histopathological findings

Liver

Liver was the primary target organ for *Piscirickettsia salmonis*, with the majority of exposed fish developing moderate to severe, necrotizing, granulomatous hepatitis (Fig. 2A–D). Principal liver lesions included: granulomas (GRN), macrophage aggregates (LMA), capsular inflammation (LCI), and focal necrosis (LFN). Foreign body-type granulomas, with central necrosis and thick layers of epithelioid macrophages, were exclusive to exposed fish. Granulomas were not observed in the high-dose group until 3 to 8 wk post-exposure when prevalence reached 80% (Table 4). In the low-dose group, there was progressive increase in granuloma prevalence and severity over time, from 0% (0 to 1 wk) to 13% (1 to 3 wk) to 44% (3 to 8 wk) (Table 4).

Macrophage aggregates and granulomatous capsulitis were observed in some control fish, but prevalences were lower compared to those in exposed fish. Over the entire course of the experiment, LMA prevalence was 83% in the high-dose group, 93% in the low-dose group and 37% in controls (Table 4). LCI prevalence in both exposure groups was ≥90%, and 66% for control fish.

Focal necrosis, another lesion directly associated with exposure to *Piscirickettsia salmonis*, was characterized by foci and areas of dead hepatocytes infiltrated by large numbers of mononuclear inflammatory cells (Fig. 2C,D). In some fish, necrosis was massive, with huge coalescing areas of dead tissue accounting for loss of up to 80% of the hepatic parenchyma. In some livers with severe necrosis, dead hepatocytes and/or macrophages were observed with clusters of intracytoplasmic, basophilic rods (presumptive *P. salmonis* bacilli). Necrotizing lesions were observed in 52% of high-dose and in 47% of low-dose fish exposed fish (Table 4). In contrast, LFN was observed in only 7% of the control fish, and necrotizing lesions were mild and limited. Among *P. salmonis*-exposed fish, LFN had a temporal pattern, with prevalence greatest during the 1 to 3 wk post-exposure period (Table 4). For example, LFN prevalence in the high-dose group increased from 17% (0 to 1 wk) to 92% (1 to 3 wk), before falling to 40% (3 to 8 wk). This same temporal pattern was also observed in the low-dose exposure group.

Additional common liver lesions included hepatocellular glycogen depletion (GD), single-cell necrosis (SCN), spongiosis hepatis (SH), and hepatocellular regeneration (HR). GD, the loss of cytoplasmic glycogen stores and increased basophilia, and SCN, random loss of individual hepatocytes, were classified as nonspecific lesions. SH, characterized by multilocular cystic cavities lined by stellate cells, and HR, or proliferation of bile preductular epithelial cells (presumptive stem cells) and immature hepatocytes, were residual or reparative lesions. All 4 were early lesions, with prevalence, in general, peaking during the first 3 wk post-exposure (Table 4).

Spleen

The 2 major splenic lesions were macrophage aggregates (SMA) and interstitial inflammation (SII). SMA

Table 4. *Atractoscion nobilis*. Temporal distribution of liver histopathology data (lesion prevalence, %) for juvenile white seabass exposed to 2 concentrations (1.99×10^4 and 3.98×10^2 50% tissue culture infective dose, $TCID_{50}$) of *Piscirickettsia salmonis* (White Seabass Isolate 98, WSB-98) via intraperitoneal (i.p.) injection. High dose, Low dose: i.p. injection with 1.99×10^4 and 3.98×10^2. $TCID_{50}$ of WSB-98 bacterium, respectively; Control: i.p.-injection with culture media fluid from uninfected CHSE-214 cells. GRN: granulomas; LMA: macrophage aggregates; LCI: capsular inflammation; LFN: focal necrosis; GD: glycogen depletion; SCN: single-cell necrosis; SH: spongiosis hepatis; HR: hepatocellular regeneration

Post-exposure time period	Liver lesion							
	GRN	LMA	LCI	LFN	GD	SCN	SH	HR
High dose								
0–1 wk (n = 12)	0	75	83	17	100	67	83	25
1–3 wk (n =12)	0	92	100	92	100	58	33	42
3–8 wk (n = 5)	80	100	100	40	60	0	20	0
Mean prevalence (n = 29)	14	83	90	52	93	48	52	28
Low dose								
0–1 wk (n = 4)	0	75	100	50	100	75	25	50
1–3 wk (n = 8)	13	100	100	100	100	63	50	100
3–8 wk (n = 18)	44	100	100	22	17	11	22	22
Mean prevalence (n = 30)	27	93	97	47	50	33	30	43
Control								
0–1 wk (n = 12)	0	50	83	8	83	50	67	25
1–3 wk (n = 12)	0	25	50	0	58	17	17	8
3–8 wk (n = 17)	0	35	65	12	29	6	24	24
Mean prevalence (n = 41)	0	37	66	7	54	22	34	20

Table 5. *Atractoscion nobilis*. Distribution of kidney, spleen, intestine and gill histopathology data (lesion prevalence, %) for juvenile white seabass exposed to 2 concentrations (1.99×10^4 and 3.98×10^2 50% tissue culture infective dose, $TCID_{50}$) of *Piscirickettsia salmonis* (White Seabass Isolate 98, WSB-98) via intraperitoneal (i.p.) injection. RMA: renal macrophage aggregates; RPD: renal protein droplets; RII: renal interstitial inflammation; SMA: splenic macrophage aggregates; SII: splenic interstitial inflammation; IGI: intestinal granulomatous inflammation. Further details as in legend to Table 4

Post-exposure time period	Kidney RMA	RPD	RII	Spleen SMA	SII	Intestine IGI	Gill GGI
High dose							
0–1 wk (n = 12)	10	10	0	33	58	67	50
1–3 wk (n =12)	92	42	17	83	92	83	83
3–8 wk (n = 5)	80	60	40	100	80	80	80
Mean prevalence (n = 29)	59	33	26	66	76	76	70
Low dose							
0–1 wk (n = 4)	25	25	50	75	75	75	0
1–3 wk (n = 8)	83	33	83	88	88	75	100
3–8 wk (n = 18)	89	50	44	94	33	78	83
Mean prevalence (n = 30)	79	43	54	90	53	77	76
Control							
0–1 wk (n = 12)	9	0	0	75	17	36	58
1–3 wk (n = 12)	18	0	0	75	17	50	64
3–8 wk (n = 17)	31	0	0	94	6	6	41
Mean prevalence (n = 41)	21	0	0	83	13	28	53

was detected in all experimental groups at similar mean prevalences (Table 5). Interstitial inflammation was characterized by infiltration of interstitial tissues by mononuclear inflammatory cells (primarily macrophages), congestion of sinusoids and larger veins by macrophages, and thickening of periarteriolar sheaths with increased numbers of macrophages. There was progressive decrease in SII prevalence from the high-dose (76%) to low-dose (53%) and control (13%) groups (Table 5). In both exposure groups, prevalence was high at exposure onset (0 to 1 wk), greatest during the middle time frame (1 to 3 wk), and declining over the last 5 wk (3 to 8 wk period) (Table 5). Among controls, SII prevalence (17%) was low and static during the first 3 wk, and then dropped to 6% during the last time frame (3 to 8 wk) (Table 5).

Kidney

Renal lesions included macrophage aggregates (RMA), protein droplet formation in proximal tubular epithelium (RPD), interstitial inflammation (RII), and colonization of mesonephric ducts by an unidentified myxozoan (possibly a *Sphaerospora* sp.). Mean prevalences for RMA, RPD and RII were all markedly higher in exposed fish (Table 5). RPD and RII were not observed in any of the control fish examined. In the high-dose group, RPD and RII showed progressive, step-wise increases in prevalence with time (Table 5). In the low-dose group, RMA and RPD increased over time.

Intestine

Foci of severe granulomatous inflammation, similar to that in the liver, were observed in intestines of experimental fish. These foci were characterized by infiltration of the lamina propria and tunica submucosa with moderate to large numbers of macrophages and lymphocytes. Macrophage aggregate formation was also evident. Similar changes were also observed in the mesenteries and serosa, with or without involvement of the tunica muscularis. Prevalence of granulomatous inflammation (IGI) was markedly higher (approx. 3-fold) in the 2 exposure groups compared with that in controls (Table 5). In the high-dose group, IGI prevalence (83%) was highest in the middle time frame (1 to 3 wk), whereas in the low-dose group, IGI prevalence (75 to 78%) was consistently elevated throughout the 8 wk sampling period (Table 5).

Gill

Granulomatous inflammation (GGI) was a common finding in the gill. When severe, inflammation was characterized by infiltration of filament and lamellar tissues, with a massive number of macrophages mixed with a smaller number of lymphocytes. Inflammation was concentrated within submucosal tissues and often filled interlamellar spaces completely (Fig. 2E). Severe granulomatous inflammation was associated with foci of necrosis, single-cell necrosis, small hemorrhages, and *Piscirickettsia salmonis*-infected cells. Infected cells (either macrophages or epithelial cells) were distinctly eosinophilic, rounded, and contained intracytoplasmic bacilli (small, short, basophilic rods; Fig. 2F). Infected cells were often very superficial (i.e. at or near the filament epithelial surface). Mean GGI prevalences were 29 to 43% higher in exposed fish (Table 5). In the high-dose group, GGI prevalence increased from 50% (0 to 1 wk) to 80% (3 to 8 wk) (Table 5). With the low-dose group, there was no inflammation during the 0 to 1 wk post-exposure period; GGI lesion prevalence (100%) peaked during the middle (1 to 3 wk) time frame (Table 5).

DISCUSSION

Piscirickettsia salmonis is associated with significant losses of farmed salmonids in Chile, Europe and Canada (Cvitanich et al. 1991, Evelyn 1992, Grant et al. 1996, Olsen et al. 1997, Rodger & Drinan 1993). *P. salmonis* was initially believed to be a pathogen only of salmonids, but more recent reports of *P. salmonis*-like organisms causing diseases similar to piscirickettsiosis among nonsalmonid fishes suggest a broader host range for the bacterium. In our study, a bacterium from white seabass originally described as *P. salmonis*-like (M. F. Chen et al. 2000) was confirmed to be *P. salmonis* by rDNA-sequencing. In addition, the isolate WSB-98 of *P. salmonis* from white seabass causes a disease and mortality in experimentally infected juvenile white seabass similar to piscirickettsiosis described in salmon.

Piscirickettsia salmonis-like organisms (PLOs) have been isolated from several marine and freshwater fishes (Mauel & Miller 2002). However, the relationships among these organisms and their association with *P. salmonis* have not been defined either by molecular or antigenic or host-range comparisons. Our study of selected ribosomal sequences of the WSB-98 isolate from white seabass indicates that it belongs in the monophyletic group of bacteria from salmonids described in the newly established family Piscirickettsiaceae (Fryer & Lannan in press). Supporting this placement, the overall rDNA sequence homology demonstrates that WSB-98 shares a closer relationship with certain isolates of *P. salmonis* (i.e. NOR-92) than those shared between isolates from salmon (i.e. LF-89 compared to NOR-92) (Tables 1 & 2). In their initial report of the isolation of the bacterium, M. F. Chen et al. (2000) described the relatively poor reactivity of WSB-98 with polyclonal antibodies to *P. salmonis* (LF-89). That antigenic differences are present among suspected *P. salmonis* isolates has been reported for salmon as well (Grant et al. 1996). Currently, the use of anti-*P. salmonis* polyclonal antibodies is described as a confirmatory test for the presence of *P. salmonis* (Office International des Epizooties 2000). With the appearance of these new isolates, further confirmatory tests, including current or newly designed PCR tests may be needed to identify certain *P. salmonis* isolates. That the WSB-98 isolate is indeed a closely related bacterium to *P. salmonis* as found in salmon was evident when it was recognized by the PCR test to detect rDNA designed by Mauel et al. (1996). In contrast, other *P. salmonis*-like organisms from nonsalmonid hosts so far tested cannot be identified by this PCR test.

WSB-98 induced both mortality and disease among juvenile white seabass injected with the bacterium in our laboratory trials at doses of 1.99×10^4 $TCID_{50}$ or 3.98×10^2 $TCID_{50}$ WSB-98 per fish. In both the high- and low-dose groups, there was little evidence of gross pathological changes associated with infection. Some infected fish had off-white, circular, opaque nodules on their liver, but most fish that died during the course of the experiment or were sampled showed no gross signs of infection. Salmonids infected with *Piscirickettsia salmonis* display a range of gross signs, including darkening, inappetance, lethargy, pale gills and skin lesions (Fryer & Hedrick 2003). However, less affected fish may show no abnormal external signs, consistent with our findings in white seabass experimentally exposed to WSB-98. While 26% (13/50) of the control fish died during this experiment, WSB-98 was never reisolated from these fish nor detected by Giemsa or immunofluorescent staining of impression smears. The parasites detected in the kidney of fish (including WSB-98-exposed fish) used in this experiment may have compromised their health and contributed to the control fish mortality. Unfortunately, pathogen-free individuals were not available for this study, as all white seabass are reared at this single hatchery.

Peak mortality in the high- and low-dose exposure groups occurred between 1 to 3 and 3 to 5 wk, respectively, and histopathological changes were detected in multiple organs. In fish surviving the experimental exposures, the bacterium was only detected in 1 fish at 60 d (Table 3, 1/38 or 2.6%), suggesting that *Piscirickettsia salmonis* is cleared reasonably quickly in fish which do not become infected. The liver was a primary target for *P. salmonis* and was one of the earliest organs involved following intraperitoneal (i.p.) injection. The spleen, kidney, intestine and gills were secondary sites of infection. Similar to our observations with white seabass, infections in salmonids induced by i.p. injection are characterized by a serosal spread. Peritoneal organ capsules are initial sites of replication, followed by parenchymal invasion (Almendras et al. 1997). Later in infection, septicemia occurs, allowing systemic spread of the bacterium, including infection of the gills. The likely pathogenesis for WSB-98 following i.p. injection was: (1) peritonitis and capsulitis of abdominal organs (including the liver); (2) invasion through the capsule and into the hepatic parenchyma; (3) infection of macrophages and hepatocytes; (4) multiplication and subsequent necrosis of hepatic tissue; (5) histiocytic inflammatory response; (6) organization of macrophages into discrete aggregates; (7) invasion of blood vessels and spread to distant sites via infected macrophages; (8) in fish with chronic infections, foreign body GRN formation. Although some portions are speculative, the severity and temporal distribution of histopathological lesions support most of this proposed pathogenesis.

The 4 liver lesions strongly linked with *Piscirickettsia salmonis* infection were foreign body GRN, LMA, LCI and LFN. While there was clear separation between control and exposed fish, there was not a clear dose–

response curve with any of the 4 lesions, which may be indicative of the virulence of WSB-98. In fact, the low-dose group had higher total lesion prevalence for 3 of the 4 major lesions (GRN, LMA, and LCI). Chronology of events was similar between the 2 exposure groups: early capsular inflammation and parenchymal necrosis, followed by macrophage aggregates, and finally granuloma formation. Direct comparison of the 2 exposure groups, however, could not be made because sampling dates and sample numbers differed greatly. With the high-dose exposure group, sampling was skewed toward early dates (n = 12 each during Weeks 0 to 1 and 1 to 3; n = 5 during Weeks 3 to 8). In contrast, with the low-dose exposure group, sampling was heavily weighted toward the end of the experiment (n = 4 during Week 1; n = 8 during Weeks 1 to 3; n = 18 during Weeks 3 to 8). The presence of some liver macrophage aggregates and capsular inflammation among control fish is not surprising. Macrophage aggregates—in small numbers and of small size—are a common finding in fish and can be sequellae to a wide range of insults, including parasites, infection and necrosis. Both the capsulitis and macrophage aggregates observed in control fish may have been secondary to chemical irritation (from the i.p.-injection vehicle).

The spleen was not a primary target organ for *Piscirickettsia salmonis*, and lesions were relatively mild (no necrosis, no granuloma formation, and mild capsulitis) in comparison to those in the liver. Inflammatory lesions were observed, but almost all were deep within the parenchyma. Similarly, the kidney was not a primary target organ for *P. salmonis*; lesions were again mild in comparison to those in the liver. The most likely pathogenesis is as follows: (1) *P. salmonis*-exposed fish develop peritonitis and liver capsulitis; (2) the liver is invaded with subsequent severe necrosis and granulomatous inflammation; (3) hepatic blood vessels are invaded and infected, then macrophages/monocytes are transported to distant sites (including spleen and kidney); (4) infected monocytes/macrophages settle out from splenic/renal capillaries and invade interstitial connective tissue of hematopoietic regions in spleen and kidney; (5) interstitial inflammation (mixed mononuclear cells) develops, with low level destruction of adjacent tissues; (6) macrophages begin to clean up the debris and infected cells; (7) macrophages form aggregates.

The intestine, similar to the spleen and kidney, was a secondary target for *Piscirickettsia salmonis*. Although there was some evidence of direct extension—through the serosa and into the tunica muscularis—of peritoneal inflammation/infection, the majority of intestinal lesions were centered in either the mucosa or submucosa and were presumed to have originated from embolic spread of the organisms via the vasculature. Granulomatous inflammation was the only intestinal lesion that correlated well with *P. salmonis* exposure and is consistent with granulomatous lesions observed in other organs.

Gills were not a primary target organ for *Piscirickettsia salmonis* (when introduced via injection exposure) but rather *P. salmonis* spread to the gill (from the liver) via infected macrophages in the blood stream. It is possible that once the gills were infected, the organisms could gain entrance into the water column (and potentially infect other fish) when dead tissue sloughed from inflamed filaments. Additional spread may come about through cannibalism, especially under crowded hatchery or aquaculture conditions. Also similar to other organs examined, granulomatous inflammation was again strongly correlated with *P. salmonis* exposure. Prevalence of granulomatous lesions was 18 to 24 % higher in exposed fish gills. Filament epithelial hyperplasia was consistently seen with granulomatous inflammation and was also strongly correlated with *P. salmonis* exposure.

In preliminary experiments, we demonstrated that the WSB-98 can be reisolated from both Chinook and coho salmon following cohabitation with experimentally infected white seabass in seawater at 16°C (unpubl. results). The rickettsial organism was reisolated on CHSE-214 cells from tissue homogenates of kidney and spleen collected from the salmon as early as 39 d after initiation of cohabitation with the white seabass. The bacterium was confirmed by IFAT using rabbit antisera specific for the WSB-98. Thus it appears that the host can shed the bacterium, which is relatively stable in seawater (M. F. Chen et al. 2000) and this may serve as an important route of fish to fish transmission.

Our studies have confirmed that a bacterium (WSB-98) from juvenile white seabass is indeed *Piscirickettsia salmonis* and that following experimental exposures WSB-98 can induce a disease with the characteristics of piscirickettsiosis in salmonid fish. We presume that further characterizations of *P. salmonis*-like bacteria found among other nonsalmonid hosts will show a spectrum of close to more distant affinities with known members of the family Piscirickettsiaceae, thus extending the host range of this emerging group of pathogens. The potential movements of isolates or strains of *P. salmonis* between different fish hosts warrants further study both to control piscirickettsiosis and as a potential explanation of the origins and spread of this important pathogen in salmonid and marine fish aquaculture.

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