Title
Control of Rickettsial Infections in White Sea Bass (Atractoscion nobilis)

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Project title: Control of Rickettsial Infections in White Sea Bass (*Atractoscion nobilis*)
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Overall Summary

This project was designed to address an emerging disease problem encountered in restoration efforts for the white seabass (*Atractoscion nobilis*) in California. Once an abundant species, declines have encouraged hatchery-based enhancement of this popular sport fish. Infections with a rickettsial-like intracellular pathogen caused serious losses in the hatchery in 1998. Losses in the hatchery exposed serious knowledge gaps with respect to the type of intracellular parasite causing the disease, the potential sources of the parasite, what diagnostic methods could be developed to detect it, what impacts could the parasite have on other marine or anadromous species (e.g., Pacific salmon) and lastly what means might be developed to control the disease caused by the parasite.

Isolation and then characterization of the intracellular bacterium proved that the bacterium was *Piscirickettsia salmonis*, an intracellular pathogen thought only to infect salmon (Fryer et al. 1992). Based on ribosomal DNA sequencing, the white seabass bacterium was as closely related to strains of *P. salmonis* from salmon in different geographic regions as the salmon isolates were to each other. The PCR assay developed for *P. salmonis* was found to be an effective molecular detection procedure for the new white seabass isolate. An enzyme linked immunosorbent assay (ELISA) was developed to detect antibodies to *P. salmonis* as found in experimentally infected white seabass. This new test provides the ability to assess prior exposures of white seabass to the bacterium. A comprehensive infectivity trial demonstrated that the bacterium isolated from white seabass when experimentally introduced into juvenile white seabass caused a disease identical to that originally observed in the hatchery losses. In addition, cohabitation of experimentally infected white seabass with juvenile chinook salmon, resulted in the transmission of the bacterium to the salmon. Lastly, approaches to control of the bacterium in white seabass can parallel those known for *P. salmonis* in salmon, which include antibiotic therapy (partially efficacious) and vaccination (currently under development). In addition, new and established detection procedures (ELISA and PCR) are being applied to determine whether the bacterium is present in wild and broodstock white seabass and potentially other marine fish species living in the lagoon where the water intake for the hatchery is located. These latter studies will aid in the design of more effective ways to avoid transmission of the bacterium to hatchery populations either by selectively breeding *P. salmonis*-free fish or preventing entrance of the bacterium in hatchery intake water.

Final Report on Specific Objectives

Objective 1: Improved Detection of the Organism

Genetic comparisons

The white seabass isolate had a 95-99% homology with the LF 89 and EM 90, NOR 92, and ATL-4-91 (isolates of *P. salmonis*) from salmon in Chile, Norway and British Columbia, Canada, respectively (Table 1). The ribosomal DNA sequences examined for the 16S, ITS-1, and the 23S regions of the four isolates were very similar but regions of homologous loci did vary between isolates as shown previously with other isolates of *P. salmonis* (Mauel et al. 1999). In a pair-wise analysis of the percent sequence similarity in which only the variable loci were considered the white seabass isolate was 67.27% similar to the type strain LF-89, 10.91% similar to EM-90, and 69.10% similar to NOR-92.

	Seabass	LF-89	EM-90
Seabass	-	-	-
LF-89	67.27	-	-
EM-90	10.91	20.0	-
NOR-92	69.10	56.36	14.54

PCR

Also, an amplicon of 467 base pairs (bp) was obtained with white seabass DNA when the PCR assay was conducted using type-strain specific primers to *P. salmonis* (Mauel et al. 1996). Sequencing of that product showed only minor bp differences from the type strain of *P. salmonis* (LF-89). These results indicated that the diagnostic PCR developed for *P. salmonis* was an adequate tool for detection of the bacterium in white seabass.

ELISA

An ELISA was developed to measure the level of anti-*P. salmonis* antibodies in the serum of white seabass. The assay has been optimized and the current protocol is as follows.

The white seabass *P. salmonis* was grown in CHSE cells and purified by density gradient centrifugation. The purified antigen was homogenized in a Bead-beater tissue homogenizer and diluted in phosphate buffer with 0.6M NaCl. This antigen preparation is used to coat ELISA plates at 4 μg/ml, 50 μl/well overnight at 4°C. The plates are blocked for 1 hour at room temperature with 2% non-fat dried milk in PBS. After blocking (and all subsequent steps) the plates are washed with PBS with 0.05% Tween-20. White seabass serum, diluted 1/400 in PBS with 0.05% Tween 20 and 1% non-fat dried milk, is added to the plate in triplicate wells, 50 μl/well. For the next step biotinylated rabbit anti-white seabass immunoglobulin is diluted 1/3200 in PBS with 0.05% Tween 20 and 1% non-fat dried milk and added to all wells of the plate at 50 μl/well and incubated for 1 hour at room temp. After washing the plate HRP-streptavidin, diluted 1:1600, is added to the plate and the plate is incubated for 30 minutes at room temperature. Finally the plate is washed and developed for 15 minutes with 100 μl/well TMB, stopped with 50 μl/well 1 M H_2SO_4. The plate is read at 450 nm.

Figure 1 shows graphically results from the white seabass serum from fish experimentally inoculated with *P. salmonis* and then sampled approximately 3 months later. Those fish known to be injected with low levels of the bacterium demonstrated elevated antibody concentrations compared to control white seabass that were not injected with the bacterium. These initial results provided evidence that there indeed is an antibody response to the bacterium following exposure of white seabass to the bacterium. We will now apply this same test to additional white seabass from experimental trials, develop a cutoff value that should separate known exposed from known unexposed fish that can then be applied to serum samples collected from wild and broodstock white seabass.

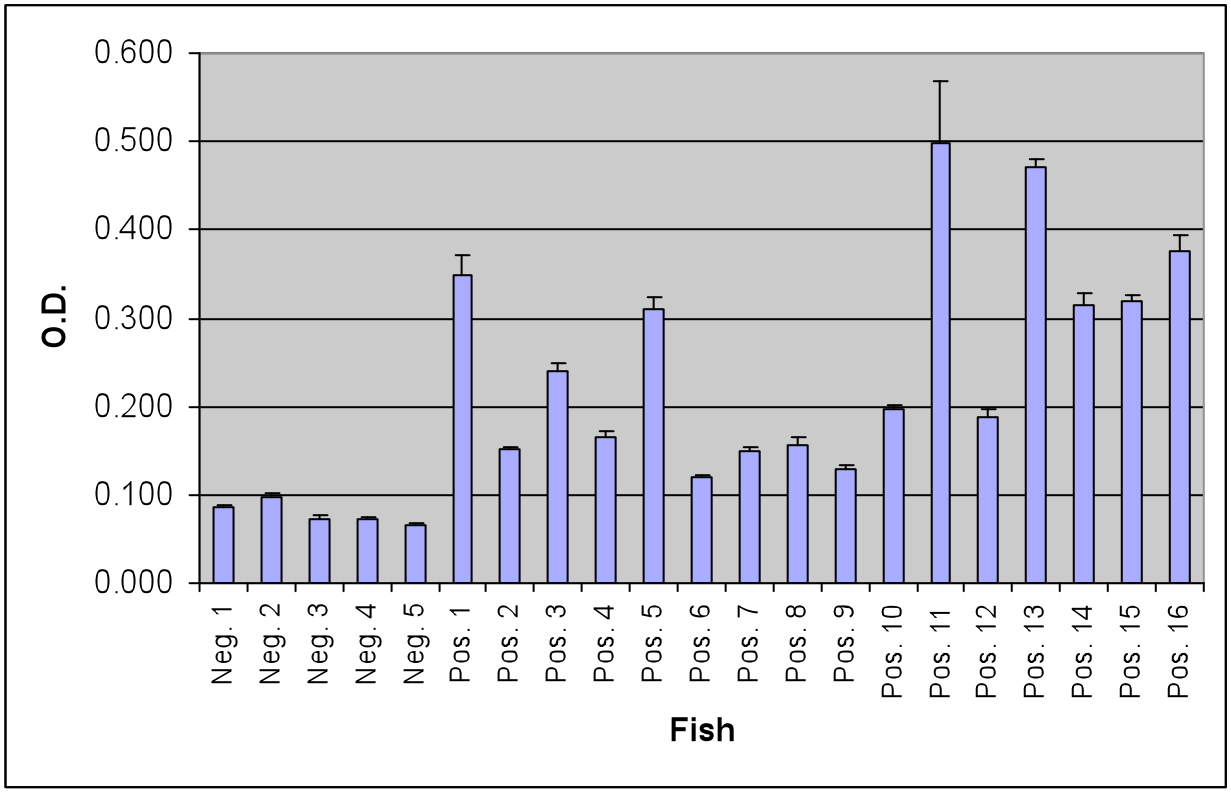

Figure 1. Concentrations of anti-*P. salmonis* antibodies detected in the serum of known exposed (Pos.) and known unexposed (Neg.) white seabass using the newly developed ELISA.

Objective 2: Steps Toward Prevention

Transmission to White Seabass – Koch's Postulates

Experimental trials were conducted to demonstrate that the bacterium isolated from the white seabass losses at the hatchery had the capability to cause disease and mortality in white seabass and Pacific salmon. A transmission trial was therefore conducted which examined the response

of juvenile white seabass injected with two concentrations (high = 1.99 x 10^4 $TCID_{50}$ or low = 3.98 x 10^2 $TCID_{50}$) of *P. salmonis*. Mortality was evaluated in one set of replicate aquaria (B Tanks) and sequential samples of live and dead fish were examined from the other replicate aquaria (A Tanks) for the extent of microscopic pathological lesions and concentrations of the bacterium in selected tissues. All dead fish were examined for the presence or absence of the bacterium including the unexposed control groups. A total of 41/50 (82%) and 20/50 (40%) of the white seabass died over a 60 d period following exposures to the high and low doses of WSB 98, respectively. A total of 13/50 (26%) of the white seabass in the control group died.

Microscopic lesions that were observed among dead fish injected with the bacterium included focal necrosis, capsule inflammation and macrophage aggregates observed in the liver, inflammation but generally much more minor changes were observed in the spleen, kidney, intestine and gill. The liver was therefore viewed as the primary target organ for the bacterium and sequential pathological changes in experimental infections characterized as follows: 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; and 8) in fish with chronic infections, foreign body granuloma formation. All of these microscopic pathological changes are similar to those observed in salmon with *P. salmonis* infections (Garces et al. 1991).

The bacterium was recovered from nearly all fish dying that were injected with the bacterium and concentrations of the parasite rose to concentrations as great as 10^6 $TCID_{50}$ per gram of tissue. The bacterium was isolated using standard procedures and CHSE-214 cells or observed using two approaches, staining of tissue imprints with Giemsa or conducting indirect immunofluorescent antibody tests (IFAT) as described by Lannan et al (1991) (Table 1). The bacterium was not isolated or observed among control white seabass that died during the study.

Table 1. Detection of *Piscirickettsia salmonis* from dead white seabass following injection exposure. The bacterium was detected in stained (Giemsa or indirect fluorescent antibody tests IFAT) in impression smears of the liver and kidney. Number positive/number sampled. ND = not determined.

Time Frame	High Dose		Low Dose		Control	
	Giemsa	IFAT	Giemsa	IFAT	Giemsa	IFAT
A Tanks:						
Sampled 0-7 d	0/12	2/4	0/4	ND	0/12	ND
Sampled 8-21 d	9/12	2/3	3/8	1/3	0/12	0/3
Sampled 22-59 d	2/6	2/3	4/19	3/7	0/17	ND
B Tanks:						
Mortality 0-7 d	1/2	ND	0/1	ND	0/10	0/7
Mortality 8-21 d	36/36	10/11	8/10	3/3	0/1	0/1
Mortality 22-59 d	2/3	1/1	7/9	¾	0/2	0/2
Sampled at 60 d	1/9	ND	0/10	0/1	0/10	ND

Transmission of *P. salmonis* from White Seabass to Pacific Salmon

Direct Injection Trials

In the first set of trials with Pacific salmon, coho and chinook salmon were injected directly with the bacterium from white seabass. White seabass were also injected as a positive control. Parallel groups of coho and chinook salmon and white seabass were injected with tissue culture media without the bacterium as negative controls. The studies were conducted in seawater at a water temperature of 15ºC. White seabass injected with the bacterium began dying within 6 days and by 8 days all 8 dead fish examined were positive for high concentrations of the bacterium (Table 2). Healthy appearing chinook and coho salmon at 14 and 28 days post injection were shown to harbor high concentrations (up to 10^4 $TCID_{50}$ per gram of tissue) of the bacterium but no mortality was observed. No *P. salmonis* was detected among chinook or coho salmon or white seabass controls not receiving the bacterium by injection. That the white seabass *P. salmonis* can cause infection and mortality in coho salmon was demonstrated in our earlier injection trials that were conducted in fresh water (Chen et al. 2000).

Table 2. Reisolation of a *Piscirickettsia salmonis* from white seabass, chinook and coho salmon following injection exposure. ND= no cytopathic effect detected.

Group	Dosage of WSB 98 (0.1 ml/fish)	Days Post Exposure	#Infected/ #Examined	Mean $TCID_{50}$ per Gram of Fish Tissue (Range)	IFAT
Exposed Seabass	1.58×10^4	6-8	8/8	6.13×10^7 (6.76×10^5 to 1.70×10^8)	+
Control Seabass	Diluent only (MEM)	28	0/3	ND	-
Exposed Chinook	1.58×10^4	14	3/3	5.4×10^4 (2.95×10^3 to 9.32×10^4)	+
Exposed Chinook	1.58×10^4	28	7/7	8.4×10^5 (1.17×10^2 to 5.67×10^6)	+
Control Chinook	Diluent only (MEM)	28	0/3	ND	-
Exposed Coho	1.58×10^4	14	3/3	3.7×10^4 (2.80×10^3 to 9.32×10^4)	+
Exposed Coho	1.58×10^4	28	4/7	2.9×10^5 (5.67×10^2 to 9.74×10^5)	+
Control Coho	Diluent only (MEM)	28	0/3	ND	-

* All exposed white seabass were collected as mortalities.

Cohabitation Trials – White Seabass to Pacific Salmon

In further experimental trials, white seabass injected with the bacterium were placed into cages suspended into aquaria containing either chinook or coho salmon. These studies were designed to demonstrate whether infected white bass could release sufficient bacterial cells to infect Pacific salmon in near proximity. These studies were conducted in seawater at a temperature of 15ºC. This trial is still in progress. Examinations of white seabass that died during the cohabitation period indicated they harbored significant numbers of bacterium, some of which presumably spread via the water to the salmon in the aquarium (Table 3). Chinook salmon used in the study underwent significant mortality due to seawater adaptation. Examination of dead chinook salmon from groups exposed to infected white seabass reveal that they have obtained infections (Table 4). Concentrations over 10^6 $TCID_{50}$ per gram of tissue have been detected in dead chinook salmon that would suggest serious infections that could have contributed to their death. We suspect that the stress associated with poor seawater adaptation has caused these chinook salmon to be more susceptible that the coho salmon to infections obtained by cohabitation with infected white seabass. To date coho salmon, which made the adaptation to seawater with fewer problems than chinook salmon, have yet to show any signs of infection.

Table 3. Concentrations of *P. salmonis* found in dead white seabass that were used as the source of the bacterium in cohabitation experiments with chinook salmon

Fish	**Date of Death Post Injection**	**Titer ($TCID_{50}$/g)**
A1-02WSB	10/4/02 (14)	2.34×10^3
A1-03WSB	10/11/02 (21)	1.07×10^5
A1-05WSB	10/23/02 (19)	1.58×10^6
B1-01WSB	10/15/02 (25)	2.34×10^5
B2-02WSB	10/16/02 (26)	1.58×10^6
B2-03WSB	10/17/02 (27)	1.58×10^6

Table 4. Results of reisolation of *P. salmonis* from chinook salmon cohabited with infected white seabass

Fish	**Date of Death Post Exposure**	**Titer ($TCID_{50}$/g)**
A1-18	10/26/02 (22 days)	$>1.58 \times 10^3$
A1-24	11/2/02	1.58×10^2
A1-31	11/9/02	2.56×10^2
A1-33	11/12/02	$>1.58 \times 10^6$
A1-35	11/14/02	1.07×10^2
A1-36	11/19/02	1.07×10^5
A1-37	11/22/02	1.58×10^6
A1-39	12/2/02	2.34×10^2
A2-28	12/2/02	$>1.58 \times 10^3$
A2-29	12/06/02	1.58×10^2

Detection in other marine fish species

Wild white seabass, Diamond turbot, California halibut and Pacific chinook salmon tissues have been examined by Dr. Martin Chen, former Fish Pathologist for the California Department of Fish and Game and an original collaborator on this project. Midway through the project, Dr. Chen relocated to Washington State where he currently is a Fish Pathologist for the Washington Department of Fish and Wildlife. He no longer is participating in the project. During his first year on the proposal he and his technical support detected PCR positive samples from California halibut, Diamond turbot, wild white seabass and chinook salmon. An amplicon of the correct weight (467 bp) was obtained from these sampled fish but we have yet to be able to corroborate those findings. We intend to sample more wild fish and will hopefully resolve this issue. ELISA results obtained from examinations of approximately 50 wild white seabass do suggest that antibody to *P. salmonis* is present in these fish which would suggest that carriers of the bacterium, that could be detected by PCR, may be present in these populations. Further testing to determine the proper positive-negative cutoff values for the ELISA following additional field sampling are underway. If the bacterium is present in fish outside the hatchery it may gain entrance through the water supply from other infected fish or alternately be present at low levels in wild broodstock. Further testing will be essential to establish these suspected routes of transmission.

Objective 3: Control Measures

Broodstock exams by PCR
Tissues from approximately 20 broodstock white seabass are presently frozen and awaiting testing. We anticipate finishing these tests and the ELISA on the serum from these fish in the next two months.

Examination of progeny
There have been no outbreaks or suspected infections among progeny in the hatchery. Recurrent problems with viral nervous necrosis (VNN) a serious neurologic disease due to a nodavirus has emerged as the dominant impediment to juvenile survival. We have begun studies to approach investigating this problem in more depth.

Antibiotic testing
The original proposal to test the antibiotic sensitivity of the unknown intracellular bacterium was discontinued when we discovered that the bacterium was *P. salmonis*. Extensive studies by both Cvitanich et al. (1991) and Fryer and Lannan (1990) have found a consistent pattern in the antibiotic sensitivity of various isolates of the bacterium, essentially showing them to be susceptible to most tested antibiotics, including those most used in aquaculture. Further testing, was therefore not deemed necessary.

Publications and Presentations

A manuscript is in preparation that describes the molecular identification (rDNA sequencing) and pathogenesis of the bacterium in white seabass. Additional manuscripts will be submitted

shortly on transmission of the agent from white seabass to salmon and development and use of the ELISA to detect antibodies in white seabass serum.

A presentation and abstract at the 4th International Symposium on Aquatic Animal Health, in New Orleans, September 2 –5, 2002 were prepared by Dr. Arkush as the first author.

References

Chen MF, Yun S, Marty GD, McDowell TS, House M, Appersen JA, Guenther TA, Arkush KD, Hedrick RP (2000) A *Piscirickettsia salmonis*-like bacterium associated with mortality of white seabass (*Atractoscion nobilis*). Diseases of Aquatic Organims 43:117-126

Cvitanich JD, Garate NO, Smith CE (1991) The isolation of a rickettsia-like organism causing disease and mortality in Chilean salmonids and its confirmation by Koch's postulate. Journal of Fish Diseases 14:121-145

Fryer JL, Lannan CN, Garcés LH, Larenas JJ, Smith PA (1990) Isolation of a rickettsiales-like organism from diseased coho salmon *Oncorhynchus kisutch* in Chile. Fish Pathology 25(2):107-114

Fryer JL, Lannan CN, Giovannoni SJ, Wood ND (1992) *Piscirickettsia salmonis* gen. nov., sp. nov., the causative agent of an epizootic disease in salmonid fishes. International Journal of Systematic Bacteriology 42(1):120-126

Garcés LH, Larenas JJ, Smith PA, Sandino S, Lannan CN, Fryer JL (1991) Infectivity of a rickettsia isolated from coho salmon (*Oncorhynchus kisutch*). Disease Aquatic Organisms 11:93-97

Lannan CN, Ewing SA, Fryer JL (1991) A fluorescent antibody test for detection of the rickettsia causing disease in Chilean salmonids. Journal of Aquatic Animal Health 3:229-234

Mauel MJ, Giovannoni SJ, Fryer JL (1996) Development of polymerase chain reaction assays for detection, identification, and differentiation of *Piscirickettsia salmonis*. Diseases of Aquatic Organisms 26:189-195

Mauel, M. J., Giovannoni, S. J., Fryer, J. L. (1999) Phylogenetic analysis of *Piscirickettsia salmonis* by 16S, internal transcribed spacer (ITS) and 23S ribosomal DNA sequencing. Diseases of Aquatic Organisms 35:115-123