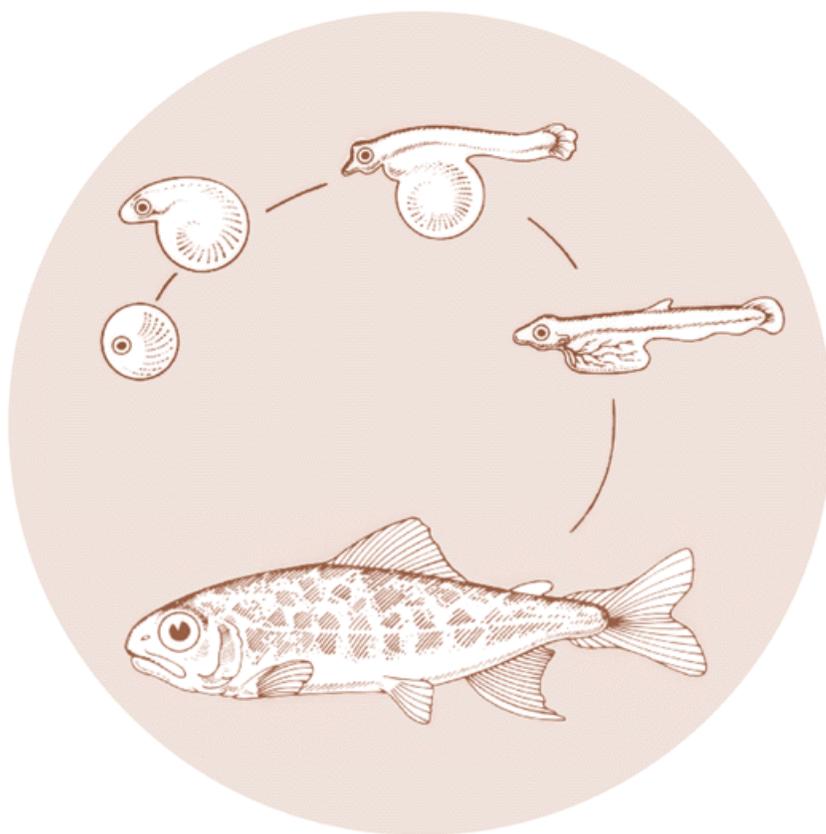


December 1990

ELISA - BASED SEGREGATION OF ADULT SPRING CHINOOK SALMON FOR CONTROL OF BACTERIAL KIDNEY DISEASE

Annual Report 1990



DOE/BP-95906-2



This report was funded by the Bonneville Power Administration (BPA), U.S. Department of Energy, as part of BPA's program to protect, mitigate, and enhance fish and wildlife affected by the development and operation of hydroelectric facilities on the Columbia River and its tributaries. The views of this report are the author's and do not necessarily represent the views of BPA.

This document should be cited as follows:

Dr. J.R. Winton, Long, J.J., Diehm, R.L., Lehner-Fournier, J.M., Kim, A. U.S. Fish and Wildlife Service - National Fisheries Research Center; Dr. S.L. Kaattari, Rockey, D.D., Dr. Wiens, G.D., Gilkey, L.L. - Dept. of Microbiology Oregon State University; ELISA - Based Segregation of Adult Spring Chinook Salmon for Control of Bacterial Kidney Disease, 1990, Annual Report 1990 to Bonneville Power Administration, Portland, OR, Contract 89FG95906, Project 89-31, electronic pages (BPA Report DOE/BP-95906-2)

This report and other BPA Fish and Wildlife Publications are available on the Internet at:

<http://www.efw.bpa.gov/cgi-bin/efw/FW/publications.cgi>

For other information on electronic documents or other printed media, contact or write to:

Bonneville Power Administration
Environment, Fish and Wildlife Division
P.O. Box 3621
905 N.E. 11th Avenue
Portland, OR 97208-3621

Please include title, author, and DOE/BP number in the request.

ELISA – BASED SEGREGATION OF ADULT SPRING
CHINOOK SALMON FOR CONTROL OF
BACTERIAL KIDNEY DISEASE

Annual Report 1990

Prepared by:

Dr. J. R. Winton
J. J. Long
R. L. Diehm
J. M. Lehner-Fournier
A. Kim

U.S. Fish and Wildlife Services
National Fisheries Research Center

Dr. S. L. Kaattari
Dr. D. D. Rockey
G. D. Wiens
L. L. Gilkey

Department of Microbiology
Oregon State University

Prepared for:

U.S. Department of Energy
Bonneville Power Administration
Environment, Fish and Wildlife
PO Box 3621
Portland, Oregon 97208

Project No. 89-031
Contract No. DE-FG79-89BP95906

December 1990

TABLE OF CONTENTS

	<u>Page</u>
INTRODUCTION	2
MATERIALS AND METHODS	
I. Standardization of ELISA Reagents to be Used in this Study	
Standardization and Analysis of Antigen	
II. Development and Standardization of ELISAs and Western Blotting Procedure	
Development of a Sensitive Western Blot Procedure for the Confirmation of Low ELISA Positive Tissue Samples	
Development of a Rapid, Field ELISA	
III. Testing of Samples from Naturally and Artificially Infected Fish	
Quantitation of RS Antigen in Hatchery Samples	
Quantitation of RS Antigen in Artificially Infected Fish	
IV. Selection and Modification of Hatcheries for Segregation Experiments	
Installation of Individual Rearing Units	11
V. Determination of BKD Infection of Tissue from Adult Spring Chinook Salmon for Brood Stock Segregation	
Distribution of ELISA Values Among Adult Spring Chinook Salmon Based on Samples of Kidney Tissue	12
VI. Determination of the Extent of Vertical Transmission from Adult Spring Chinook Salmon to Progeny.	
Testing of Eggs.	16
VII. Tagging of Smolts and Evaluation of Returns from Experimental Groups.	
Investigation of Funding.	18

	<u>Page</u>
RESULTS AND DISCUSSION	
I. Standardization of ELISA Reagents to be Used in this Study	
Standardization and Analysis of Antigen	18
II. Development and Standardization of ELISAs and Western Blotting Procedure	
Development of a Sensitive Western Blot Procedure for the Confirmation of Low ELISA Positive Tissue Samples	22
Development of a Rapid, Field ELISA	29
III. Testing of Samples from Naturally and Artificially Infected Fish	
Quantitation of RS Antigen in Hatchery Samples	31
Quantitation of RS Antigen in Artificially Infected Fish	33
IV. Selection and Modification of Hatcheries for Segregation Experiments	
Installation of Individual Rearing Units	36
V. Determination of BKD Infection of Tissue from Adult Spring Chinook Salmon for Brood Stock Segregation	
Distribution of ELISA Values Among Adult Spring Chinook Salmon Based on Samples of Kidney Tissue	37
VI. Determination of the Extent of Vertical Transmission from Adult Spring Chinook Salmon to Progeny.	
Testing of Eggs.	43
VII. Tagging of Smolts and Evaluation of Returns from Experimental Groups.	
Investigation of Funding.	45
LITERATURE CITED	47

APPENDICES

	<u>Page</u>
Appendix One. Monoclonal and Polyclonal Antibody-Based ELISA Protocol.	A1
Appendix Two. Standard Western Blot Protocol.	A3
Appendix Three. ELISA Values for Adult Female Chinook Salmon at Carson National Fish Hatchery.	A5
Appendix Four. Optical Density Values for Supernatants from Eggs at Marion Forks Hatchery.	A8
Appendix Five. Publications and Presentations.	A12

LIST OF FIGURES

	<u>Page</u>
Figure 1. Comparison of production of protein by media and concentration method.	20
Figure 2. Comparison of protein activity by polyclonal antibody-based ELISA for two extraction methods and several of their intermediate steps.	21
Figure 3. Western blot analysis of the distilled water extraction method for production of soluble antigen to <u>Renibacterium salmoininarum</u> .	23
Figure 4. Western blot analysis of the ultrafiltration extraction method for production of soluble antigen to <u>Renibacterium salmoininarum</u> .	24
Figure 5. Comparison of Western blot substrates for detecting RS antigen in kidney tissue samples.	26
Figure 6. Examination by Western blot of kidney tissue samples from spring chinook salmon testing high and low by ELISA.	28
Figure 7. Comparison of sensitivity of detection of the field ELISA and the monoclonal antibody-based ELISA.	30
Figure 8. Distribution of mean optical density values for artificially infected chinook salmon.	34
Figure 9. Effects of oral erythromycin administration on concentrations of RS antigen in juvenile salmon.	35
Figure 10. Levels of RS antigen determined by the monoclonal antibody-based ELISA for adult chinook salmon selected for segregation at Marion Forks Hatchery.	38
Figure 11. Percent occurrence of optical density values determined by polyclonal antibody-based ELISA for adult chinook salmon at Carson National Fish Hatchery.	40
Figure 12. Number and mean optical density values determined by the polyclonal antibody-based ELISA for adult male chinook salmon at Carson National Fish Hatchery.	41

	<u>Page</u>
Figure 13. Distribution of mean optical density values determined by polyclonal antibody-based ELISA for adult female chinook salmon selected for segregation at Carson National Fish Hatchery.	42
Figure 14. Number and mean optical density values determined by polyclonal antibody-based ELISA for adult female chinook salmon at Carson National Fish Hatchery.	44
Figure 15. Assessment of nonspecific binding of supernatant from eggs for the monoclonal antibody-based ELISA.	46

LIST OF TABLES

	<u>Page</u>
Table 1. Steps in the monoclonal antibody-based field ELISA.	7
Table 2. Comparison of the monoclonal antibody-based ELISA, field ELISA, and direct FAT.	32

ABSTRACT

Bacterial kidney disease (BKD), caused by Renibacterium salmoninarum (RS), is a serious disease of salmonid fish worldwide. The disease has a major impact on spring chinook salmon populations in the Columbia River system. There is strong evidence that RS can be transmitted from parent to progeny, and segregation of progeny based on levels of antigen detected in adult fish may obviate this mode of transmission.

Results from the second year of a four year study to investigate segregation of broodstock as a tool for controlling BKD are presented. To segregate the progeny of adult fish infected with RS we have used enzyme-linked immunosorbent assays (ELISAs) optimized in the first year of this project. Gametes from fish either injected with erythromycin or receiving no antibiotic injection were successfully segregated into groups having either high or low levels of the RS soluble antigen. Screening of eggs from infected adults has not revealed any detectable antigen present in the egg tissue.

Development of a rapid, field ELISA has been accomplished this year. The field ELISA utilizes monoclonal antibodies (MAbs) currently employed in the monoclonal antibody-based ELISA (monoclonal ELISA). The sensitivity of the field ELISA approaches that of the monoclonal ELISA, and has been tested on 150 adult chinook salmon. A high correlation exists between samples assayed by the monoclonal ELISA, field ELISA, and direct FAT.

An alternative system for detecting RS soluble antigen, the Western blot, has also been improved. Using a chemiluminescent substrate, the sensitivity of detection has been increase 50-100 fold.

INTRODUCTION

Bacterial kidney disease (BKD) is responsible for major losses of intensively cultured salmon and trout (Oncorhynchus spp.) in the Pacific Northwest and worldwide (Bullock and Herman 1988; Fryer and Sanders 1981). The disease is caused by a fastidious, gram positive bacterium, Renibacterium salmoninarum (RS), that produces a chronic, systemic infection. Salmonids are susceptible at all life stages in both freshwater and marine environments (Banner et al. 1986). Vaccination has not been effective in controlling the disease and current treatment methods rely on antibiotic therapy, principally erythromycin, and other management strategies (Elliot et al. 1989). Control is complicated by the survival of RS within host cells and within unfertilized and fertilized eggs (Young and Chapman 1978; Evelyn et al. 1984). Intracellular survival and the presence of bacteria within the egg are viewed as contributing to bacterial persistence and vertical transmission, respectively.

This report presents the results of the work completed during the second year of a four year study evaluating the use of enzyme-linked immunosorbent assay (ELISA) as a procedure supporting the segregation of broodstock as a means for controlling BKD in hatchery situations. The major portion of this year's work was devoted to determining the levels of RS soluble antigen present in spawning chinook salmon (Oncorhynchus tshawytscha) by ELISA, selection of experimental groups of broodstock based on ELISA results, and individual rearing of eggs

and fry from single matings. Progress and development of tasks started in the first year of the study are also presented.

MATERIALS AND METHODS

I. Standardization of ELISA Reagents to be Used in this Study

Standardization and Analysis of Antigen. Methods for obtaining optimum production and quality of soluble antigen were examined using RS strain D6 (isolated from coho salmon, Oncorhynchus kisutch) grown in three different culture media: KDM-2 plus serum (KDM2-S; Evelyn 1977); KDM-2 without serum (KDM2-NS); and KDM-2 plus charcoal (KDM2-C; Daly and Stevenson 1985). Five ml of a D6 starter culture was inoculated into 100 ml of KDM2-NS and incubated with constant stirring for 19 days at 15°C to obtain an optical density (OD) of approximately 1.0. One liter of each medium was then inoculated with 5 ml of the starter culture and grown with stirring for 16 days at 15°C. Cells were separated from the medium by centrifugation at 5858 x g for 30 min at 4°C.

Soluble antigen remaining on the cells grown in each medium was removed with the modified distilled water extraction technique of Daly and Stevenson (1990). Cells were resuspended in 100 ml phosphate-buffered saline (PBS; 10 Mm PO₄, 0.85% NaCl, pH 7.2) and centrifuged for 30 min at 6000 x g and 4°C. The supernatant was decanted and the pellet extracted in 100 ml distilled-deionized water for one hour at 4°C. The suspension was centrifuged and the supernatant decanted and stored at -70°C.

This supernatant was concentrated using ultrafiltration and ammonium sulfate precipitation as described in the following paragraph.

The antigen in the supernatant from each medium was concentrated using a Minitan Ultrafiltration System fitted with two Minitan No. PLGCOMP04 (Millipore Corp., Bedford, MA), 10,000 molecular weight exclusion size, low protein-binding filter packets . After ultrafiltration, the remaining fluid was concentrated with ammonium sulfate using the protocol of Turaga et al. (1987), with the following modifications. The powdered ammonium sulfate used in the first 50% concentration was added over a two hour period and stirred an additional two hours. Following centrifugation (1464 x g for 10 min) the pelleted precipitate was resuspended in 10 ml PBS. The second 50% concentration added the saturated ammonium sulfate solution over 1 hour and the mixture was stirred for an additional hour. The pellet from the second precipitation was resuspended in five ml of PBS. The concentrate was dialyzed (Spectrapor #2; Spectrum Medical Industries, Los Angeles CA) against one liter of PBS overnight. One percent thimersol was added (10 μ l/ml) as a preservative.

Samples were taken at several stages during the concentration process to determine the total protein and the residual amounts of RS antigen by Western blot and polyclonal antibody-based ELISA (polyclonal ELISA). These included a sample

of filtrate from the Minitan, supernatant from the first ammonium sulfate concentration, and remaining supernatant from the second ammonium sulfate precipitation in the distilled water extraction.

The protocol used for analysis of the antigen by polyclonal ELISA is outlined in Appendix 1, while the protocol for the Western blot is presented in Appendix 2. Total protein concentration was determined by the Lowry method (Lowry et al. 1951) using a commercially available Protein Assay Kit Procedure No. P 5656 (Sigma Diagnostics, St. Louis, MO).

II. Development and Standardization of ELISAs and Western Blotting Procedure

Development of a Sensitive Western Blot Procedure for the Confirmation of Low ELISA Positive Tissue Samples. Kidney tissues were collected from spawning chinook salmon during the course of the segregation experiments. Concentrations of RS soluble antigen were determined using the monoclonal antibody-based ELISA (monoclonal ELISA; Appendix 1). Polyacrylamide gel electrophoresis and Western blot analysis of the kidney samples was conducted using tissues stored at -20°C . After transfer to nitrocellulose, blots were blocked in 1% BSA, incubated in monoclonal antibodies (MAb) 4D3 (Wiens and Kaattari 1989) and 3H1 (Rockey et al. in press) ($1\ \mu\text{g}/\text{ml}$ each) and then in a second antibody of goat anti-mouse peroxidase conjugate (1/1000; Hyclone Laboratories, Ogden UT) as described by Rockey et al. (in press). At this step, identical blots were either developed with a standard chromogenic substrate (4-chloro-naphthol) or developed

with a fluorogenic substrate (Enhanced Chemiluminescence reagents; ECL; Amersham Corp., Arlington Heights, IL). For the ECL substrate, blots were washed 5X for 5 min in TTBS and finally 2X in TBS. Blots were then moved to a photographic darkroom and placed in the ECL reagents (1 ml each reagent per 9 x 12 cm blot). After one minute incubation, the blots were removed, placed inside a colorless plastic "report holder", and exposed to X-ray film for a selected time interval (2 sec to 5 min). The film was then developed using standard X-ray film development procedures and dried.

The approximate sensitivity of the ECL system was determined in two ways. First, kidney tissue from a fish having high levels of RS soluble antigen was suspended 1:2 (wt/vol) in phosphate buffered saline (150 mM NaCl, 10 mM PO₄, pH 7.2) and the solids removed by centrifugation. Serial dilutions of this supernatant (56 µg/ml) were made using supernatant from a kidney sample from a fish having very low levels of antigen. A 4 µl sample of each dilution was loaded per lane of a polyacrylamide gel. The second experiment used samples prepared for the monoclonal ELISA as antigen for the blotting. The results from the blotting were then compared to antigen concentrations as determined by the monoclonal ELISA.

Development of a Rapid, Field ELISA. The protocol for this assay is outlined in Table 1. Briefly, a cotton swab was inserted into the kidney of the fish in the manner used for fluorescent antibody sampling. Swabs were placed in 5 ml polystyrene tubes which had been precoated with MAb 4D3. After

Table 1. Steps in the monoclonal antibody-based field ELISA.

Reagent	Volume (Conc)	Incubation Conditions	Incubation Time	TBBS Wash
MAb 4D3 in PBS	500 μ l (3.2 μ g/ml)	17°C	overnight	No
BSA in TBBS	4.0 ml (0.01 g/ml)	ambient temp	30 minutes	No
Sample	Swab in 500 μ l BSA-TBBS	ambient temp	30 minutes	5x
MAb 3H1 in PBS	500 μ l (1 μ g/ml)	ambient temp	30 minutes	5x
SA-HRPO in PBS	500 μ l (0.25 μ g/ml)	ambient temp	30 minutes	5x 1X TBS
Substrate	500 μ l ABTS	ambient temp, dark	10 minutes	No

the 30 min incubation, swabs were removed, tubes were washed, and a second antibody (biotinylated 3H1) applied for 30 min. The second antibody was subsequently removed and streptavidin-conjugated horseradish peroxidase (SA-HRPO) added for 30 min. Substrate was added and incubated for 10 min. Positive samples were identified by visual comparison to a reference standard. In order to compare the field ELISA, monoclonal ELISA, and FAT, a total of 150 adult chinook salmon were tested.

III. Testing of Samples from Naturally and Artificially Infected Fish

Quantitation of RS Antigen in Hatchery Samples. Samples of fry (90 fish) and yearling chinook salmon (14 fish) from Carson National Fish Hatchery were collected on Feb 2, 1990 to determine the levels of RS antigen by polyclonal ELISA. Methods for obtaining, preparing, and testing of tissues from fry and juvenile fish were assessed to determine the best approach for assaying experimental fish during the segregation experiment.

Fry were segregated into three groups of 30 fish each with three fish pooled for a sample. One group was processed as whole fish. Fry in the second group were dissected to assay different anatomical sections and processed as 1) head, back, and kidney: and 2) stomach and associated tissue. The third group was 1) head; 2) back and kidney: and 3) stomach and tail. Portions were selected to test levels of antigen in distinct body regions and to ascertain which combination of tissues would provide adequate sample for ELISA testing. Samples from juvenile fish consisting

of kidney and spleen were tested to determine what levels of antigen might be expected in yearling fish. Fish were held at - 20°C then were processed and tested by ELISA.

Quantitation of RS Antigen in Artificially Infected Fish. An experiment to determine if the results from the polyclonal ELISA were adversely affected by antibiotic treatment was conducted in Seattle using approximately 1400 fall chinook salmon. These fish were obtained from the Washington Department of Fisheries Green River Hatchery on April 3, 1990 at a size of approximately 200 fish/pound (2.5 g each). Because obtaining fish which were free of RS infection was not possible, a separate stock tank was maintained at the Seattle Laboratory from which samples of 60 fish were processed and tested by ELISA on arrival and at 51 days to establish baseline levels of RS soluble antigen.

Six experimental groups, and a replicate series, were stocked at 100 fish/tank and acclimated for 7 days at 8°-12°C. Three of the experimental groups were given intraperitoneal injections with viable RS (strain D6, grown in KDM-2-C for 16 days, suspension OD 0.114). These three groups received 1) intraperitoneal injection with erythromycin (20 mg/kg body weight), one day prior to injection of RS; 2) pelleted food containing erythromycin (4% Gallimycin fed at 2% body weight for 21 days) starting one day before injection of RS; and 3) no antibiotic administered. The remaining three experimental groups were intraperitoneally injected with sterile medium (KDM-2-C) and

received the same treatment as the above groups. Water temperature ranged from 8^o-15^oC, averaging 11^oC, during the experiment.

Following injection with RS or medium, fish from each tank were sampled at 3, 7, 14, 21, 28, 42, 56, and 70 days post-injection. Kidney tissues from fish dying during the experiment were examined by FAT to substantiate presence of RS.

A sample of kidney tissue was removed from each fish and processed for polyclonal ELISA with the following modifications. The tissue was partially diluted in PBS without Tween-20, homogenized, and a 10 μ l inoculum was removed and placed into 100 μ l of sterile PBS. The inoculated fluid was dispensed and dispersed onto KDM-2-C culture plates (1.5% agar), supplemented with 1% spent medium (Evelyn et al. 1990), and incubated at 15^oC. Plate counts were made at 20-28 days. Original tissue samples were then diluted with additional PBS-Tween 20, to reach a final dilution of 1:8.

At Oregon State University, four treatment groups were used to assess the effects of erythromycin on the levels of antigen to RS. The four treatments were: 1) fish fed Oregon Moist Pellet (OMP) containing erythromycin for seven days prior to challenge and seven days post challenge; 2) fish fed OMP containing erythromycin for 21 days post challenge; 3) fish fed OMP without erythromycin and challenged; and 4) fish fed OMP without erythromycin and not challenged. Fish receiving erythromycin were fed at 2% body weight per day with OMP containing erythromycin at 100 mg/kg. Fish were challenged with by

intraperitoneal injection of 10 μ l of washed RS cells (1×10^7 colony forming unit (cfu)/fish). Three fish per tank were sampled weekly for six weeks. Replicate tanks were used for each treatment.

IV. Selection and Modification of Hatcheries for Segregation Experiment

Installation of Individual Rearing Units. Ninety-nine individual rearing tanks were installed at Carson Hatchery to hold eggs from individual spawning. Banks of 12-13 tanks were used to hold the four experimental groups in replicate. Original plans had been to install 80-88 tanks, based on historical fecundity of returning female fish and to accommodate stocking densities required by the hatchery. The average fecundity for females returning in 1990 was lower than in the past, and egg numbers were reduced in the female fish with high levels of RS soluble antigen, necessitating larger numbers of fish in each group.

Tanks were molded fiberglass, painted with latex enamel, 15 inches square by 20 inches deep, and held approximately five gallons of water when installed. Throughout the experiment, each tank will be individually supplied with spring water (averaging 8°C), as will the experimental raceway groups.

A subsample of 250-300 eggs from each female selected for the experiment was placed into an individual tank. Fish will be held as long as possible in these tanks and sampled periodically by polyclonal ELISA for detection of antigen to RS.

At Marion Forks Hatchery, 80 individual rearing units were installed. All progeny from each adult cross were placed in separate buckets where the yolk-sac fry will remain until first feeding. Each treatment group will then be randomly divided into triplicate tanks which will contain a total of 15,000 fish/tank.

V. Determination of BKD Infection of Tissue from Adult
Spring Chinook Salmon for Brood Stock Segregation

Distribution of ELISA Values Among Adult Spring Chinook Salmon Based on Samples of Kidney Tissue. Adult chinook salmon were spawned at weekly intervals beginning August 7, 1990 at Carson Hatchery. Processing and production methods currently utilized at the hatchery were adjusted to integrate this study and minimize its impact on customary practices. Fish used in this segregation experiment represented a portion of the hatchery production and were handled accordingly. Adult chinook salmon began returning to Carson Hatchery on May 10, 1990. Fish were confined in holding ponds from their arrival until spawned or discarded. Returns continued at the hatchery through spawning, which was completed in late August.

On June 13, 1990, 1158 returning adult fish were examined, uniquely tagged with colored t-bar tags, injected with erythromycin, and returned to the holding pond. A corresponding sample of 200 adult fish was tagged and did not receive the antibiotic injection. On July 11, 1990, 567 fish entering the hatchery since June 13 were tagged and injected. A second injection was administered to previously injected adults. At

this time, 203 additional fish were marked as receiving no injection. Fish returning after July 11 possessed no mark and received no injection. This procedure was done to provide a spectrum of the returning adults to be used as experimental fish.

During the month of August, fish were checked at weekly intervals for ripeness and returned to the holding pond if green, or spawned if ripe. A total of 1080 females and 797 males were spawned. Fewer males were used due to the standard practice of fertilizing multiple females with the milt of one male.

Adult fish were anesthetized, selected for spawning, and labeled with an individual number. Tag information, representing antibiotic treatment and timing of return to the hatchery, was recorded at this time.

Both males and females were killed by cerebral concussion. Females were bled by severing the caudal artery. Eggs from a single female were collected, held separately, fertilized using the milt from one male, and transported to the brood facility at the hatchery. Contamination between females was avoided by having the person spawning the female wear a new disposable glove for each fish and by thoroughly disinfecting all common equipment with iodophor between fishes. For each family of fertilized eggs, a sample of male kidney and female kidney and ovarian fluid was collected from the two parental fish for testing by polyclonal ELISA.

On arrival in the hatchery building, the eggs were rinsed in water to remove any extraneous tissue, hardened by immersion in

75 ppm iodophor for 20 min, and placed into racks with a separate container and water supply for each individual family of eggs. Eggs from each family group were held in isolation from the time of spawning until tissues had been tested by polyclonal ELISA for the presence of antigen to RS. At this time, experimental families were selected based on the results of the ELISA testing.

Tagging information for each fish provided data on antibiotic treatment and timing of return to the hatchery. ELISA values obtained from kidney tissues of both parents were used to allocate eggs from individual spawning pairs to either low or high groups. The ELISA value for the female parent was dominant for assignment to low or high groups. Male ELISA values were utilized only to exclude eggs from matings of a low female with a high male.

Optical density data from polyclonal ELISA and information on antibiotic treatment of adults were used to apportion eggs from individual matings into four experimental groups: 1) no antibiotic injection/low ELISA (OD <0.085) (UNLO); 2) no antibiotic injection/high ELISA (OD >0.100) (UNHI); 3) antibiotic injection/low ELISA (OD < 0.085) (INLO); and 4) antibiotic injection/high ELISA (OD >0.100) (INHI). Fish were selected for the above groups based on timing of return to the hatchery: 1) May 10-June 13; 2) June 13-July 11; 3) July 11-spawning date.

Adult chinook salmon returning to the Minto collection facility of Marion Forks Hatchery typically remain in the North Santiam River until spawning and are not injected with

erythromycin. To accommodate the design of this project 450 adults were allowed to enter the facility early and were injected once with erythromycin (100 mg if fish weighed less than 18 lbs and 200 mg if fish weighted more than 18 lbs). Fish were injected 9-21 days prior to spawning. Fish were spawned using delayed fertilization techniques. Eggs were stored in zip-lock plastic bags while the milt was stored in plastic containers. On the same day that fish were spawned, kidney samples were removed, transported to Oregon State University, and assayed by the monoclonal ELISA. Kidney samples were mixed 1:2 (wt:vol) with 1% BSA-TTBS and homogenized with rolling pins. The homogenates were centrifuged (1200 x g, 5 min) and the supernatant removed and tested by ELISA. Based on results from the monoclonal ELISA, crosses between fish were completed on the following day. Sixty-nine crosses were made to provide families that could be distributed among four treatment groups as follows: 1) not injected with erythromycin and low ELISA (N=19); 2) not injected and high ELISA (N=19); 3) injected with erythromycin and low ELISA (N=20); and 4) injected and high ELISA (N=11). A total of 440 fish were screened to obtain the fish necessary for the 69 crosses.

Samples were tested with monoclonal ELISA using the procedure described in Appendix 1. Supernatant from tissue was loaded directly onto the plate. All manipulation of reagents, excepting the addition of the samples from the tissues to be

tested for antigen, was performed with an Autodrop Plate Loader (Flow Laboratories, McLean, VA). Replicates or duplicates of standard dilutions of antigen (50 μ l each plate; 1-25 ng/ml) and replicates of dilutions of tissue samples were loaded onto the plates (50 μ l/well) using hand-held pipetters. All washes were performed using a Titertek Microplate Washer 120 (Flow Laboratories) with TTBS as the wash buffer. Streptavidin-conjugated-horseradish peroxidase was produced commercially (Sigma Chemical Co., St. Louis, MO). Optical densities (A_{405}) were quantitated on a Titertek Multiskan Plus Microplate Reader (Flow Laboratories).

Regression analysis permitted the derivation of the line of best fit from standard curves and calculation of correlation coefficients were derived using a graphics program (Cricketgraph, Cricket Software, Philadelphia, PA). The concentration of antigen in each tissue sample was calculated from the average optical densities using this linear equation. Dilutions were performed on highly positive tissues to reflect samples with optical densities within the standard curve. Data from the standard curve with correlation coefficients of less than 0.96 were not used for calculations.

VI. Determination of the Extent of Vertical Transmission

Testing of Eggs by ELISA. At Carson Hatchery, a sample of 30 eggs was collected from each of the 99 individual tanks approximately 69 days following fertilization. Average daily thermal unit (DTU) value for these eggs was 900 and water

temperature averaged approximately 7^o-8^oC . Eggs were held at 4^oC and were processed on the day of collection or on the following day. Processing consisted of adding PBS at 1:5 (wt/vol), puncturing the egg, and mixing the contents by vigorous agitation. Processed homogenates of eggs were stored at -70^oC until testing by polyclonal ELISA. Protocol for the ELISA followed the established protocol, excepting the exclusion of the boiling of egg tissues prior to testing.

Fertilized eggs from adult chinook salmon at the Marion Forks facility were screened by the monoclonal ELISA 60-62 days after fertilization. Eggs had reached approximately 700 DTU at the time of screening. Eggs were washed with distilled-deionized water, placed in Eppendorf tubes, punctured with 18 gauge needles, and homogenized. Egg samples were diluted 1:2 with 1% BSA-TTBS and vortexed before being loaded onto the ELISA plate. Because of potential substances in the egg which might increase background in the ELISA, a further control was added to the assay. This additional control consisted of using a purified MAb MOPC 21 (Sigma), which does not recognize RS antigens, as a coating antibody. Optical density was compared between wells coated with 4D3 and wells coated with MOPC 21.

To determine if antigen in the egg might be sequestered by egg components, samples of eggs from both low and high parents were spiked with dilutions of the antigen used for the standard curve. Samples were subsequently analyzed by the monoclonal ELISA.

VII. Tagging of Smolts and Evaluation of Returns from Experimental Groups.

Investigation of Funding. Discussions with various agencies and individuals indicated strong support for the tagging and analysis of returning adults as part of this study. Originally included as an optional objective of the project, obtaining funds for the tagging of all experimental fish was deemed necessary to provide adequate numbers of returning adult fish from each of the experimental groups. Several sources of funding have been contacted and discussions continue to secure funding for tagging and monitoring at each of the facilities for both years of the segregation analysis.

RESULTS AND DISCUSSION

I. Standardization of ELISA Reagents

Standardization and Analysis of Antigen. The comparison of six culture media for maximizing growth and production of antigen and two strains (ATCC 33209 and D6) of RS was completed in the first year of this study (Kaattari et al. 1989). Growth of strain D6 surpassed that of ATCC 33209 for all but one of the media tested. Analysis of antigen by Western blot showed that the 57 kDa protein (antigen F; Getchell et al. 1985) predominated in growth media containing serum. The presence of serum in the growth medium was the major factor affecting the quality of the antigen and breakdown products of the 57 kDa antigen were found in media lacking serum. Although growth in media containing

serum produced a large amount of the 57 kDa protein, total protein determination of these preparations reflected the serum from the medium together with proteins originating from RS.

Since the construction of a standard curve for the quantitation of antigen in the ELISA relies on the use of purified antigens of RS, production of consistent, well-characterized antigen is essential. The method of producing antigen must yield dependable results, be economical, and the technology should be readily transferrable to other laboratories.

Several methods for evaluating the production and refining the quality of antigen produced by RS, strain D6, were investigated. This strain was chosen as it is pathogenic for both coho and chinook salmon and grows well under controlled conditions. Based on the results from work in the first year of the project, KDM-2 was selected as the most appropriate medium to optimize growth and maximize production of antigen.

Extraction with distilled water yielded the lowest amount of total protein while ultrafiltration yielded the highest amounts of protein for the three media tested (Figure 1). Concentration of soluble antigen, determined by polyclonal ELISA was higher for antigen extracted by distilled water from cells grown in KDM2-S than from cells grown in KDM2-C or KDMZ-NS, but the activity was relatively equal for soluble antigen produced with ultrafiltration (Figure 2).

The Western blot of the soluble antigen extracted with distilled water, indicated growth in KDM2-C and KDMZ-NS produced

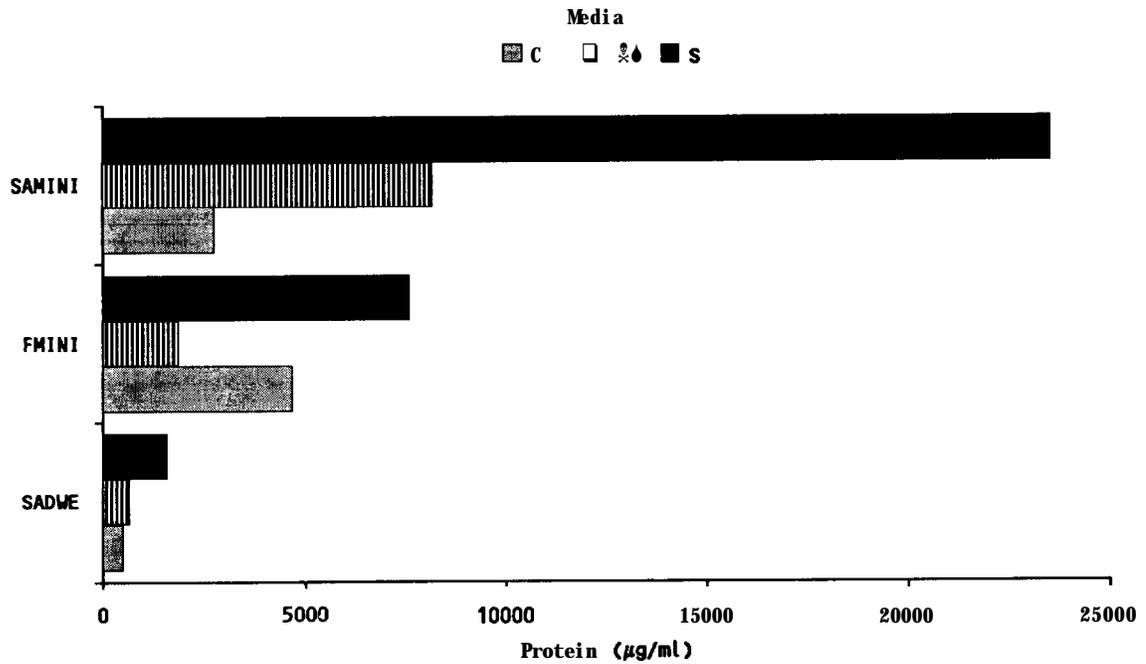


Figure 1. Comparison of production of protein for three media by Lowry assay. (Abbreviations: C=KDM2-C, NS=KDM2-NS, S=KDM2-S, SADWE=Soluble antigen obtained from distilled water extraction, FMINI=Filtrate from ultrafiltration, SAMINI=Soluble antigen obtained from ultrafiltration).

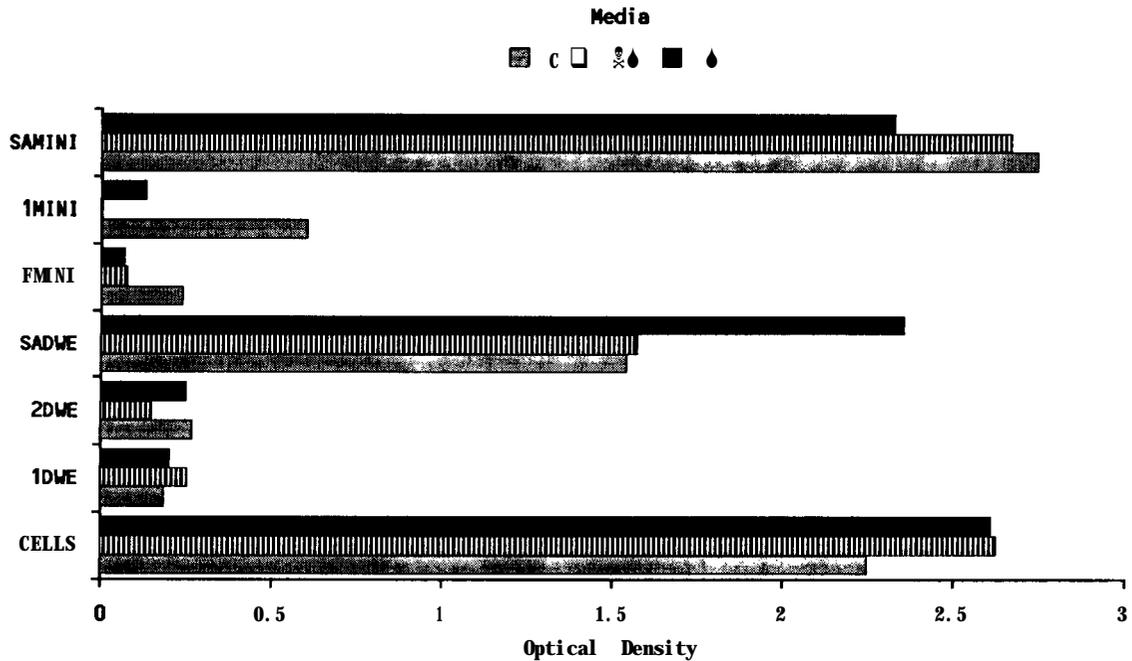


Figure 2. Comparison of activity, determined by polyclonal antibody-based ELISA, of protein for three media obtained from distilled water extraction, ultrafiltration, and several intermediate steps. (Abbreviations: C=KDM2-C, NS=KDM2-NS, S=KDM2-S, CELLS=Cells remaining after distilled water extraction, 1/2DWE=Supernatant remaining from first and second SAS concentrations of distilled water extraction, SADWE=Soluble antigen obtained from distilled water extraction, FMINI=Filtrate from ultrafiltration, 1MINI=Supernatant remaining from first SAS concentration of ultrafiltration, SAMINI=Soluble antigen obtained from ultrafiltration).

the highest purity antigen (Figure 3). While ultrafiltration yielded the highest amount of protein for the three media tested (Figure 1). Western blot of soluble antigen extracted by ultrafiltration indicated cells grown in KDM2-S had the most stable 57 kDa antigen as the typical breakdown products of the 57 kDa antigen were observed when cells were grown in KDM2-C and KDM2-NS (Figure 4).

This preliminary analysis indicates that the ultrafiltration method yields the highest quantity and most active soluble antigen. Subsequent analysis will be directed at refining this technique for the production of soluble antigen to be used as a positive control for ELISA.

II. Development and Standardization of ELISAs and Western Blotting Procedure

Development of a Sensitive Western Blot Procedure for the Confirmation of Low ELISA Positive Tissue Samples. Detection methods for RS have grown increasingly sensitive as new technical developments occur. Currently, the techniques with the highest sensitivity are the ELISA and, possibly, rigorous culture. In many laboratory situations, this increased sensitivity has led lightly infected fish being identified as positive for RS by ELISA, but this cannot be confirmed by other detection techniques. One possible tool that may be useful for the verification of these samples is Western blotting.

Western blotting is a procedure which combines the electrophoretic separation of proteins with immunodetection

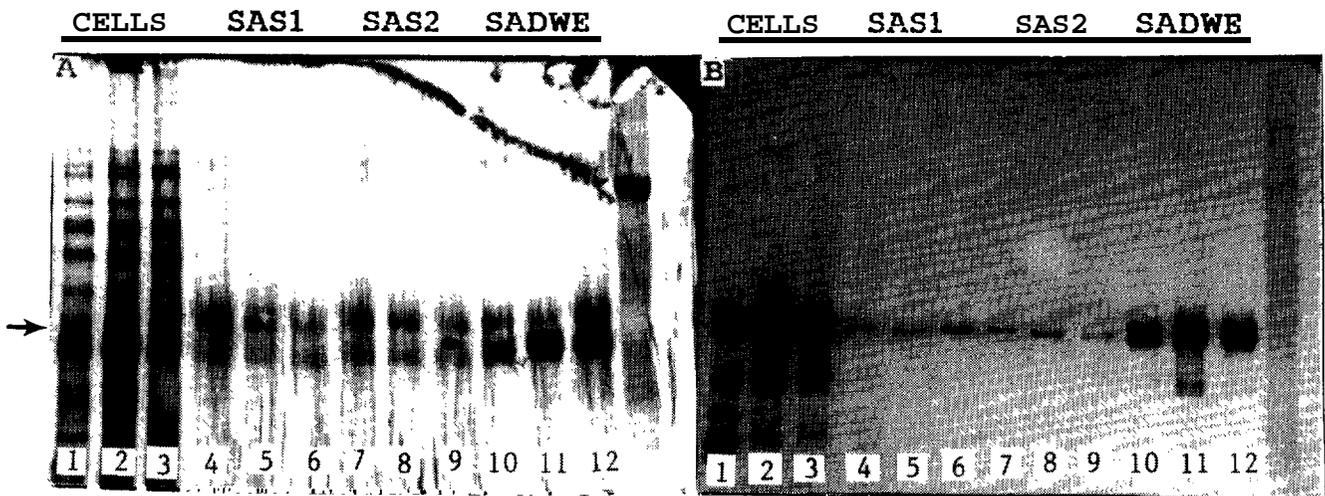


Figure 3. Western blots comparing distilled water extraction for the production of soluble antigen from Renibacterium salmoninarum. A) Total protein stained with colloidal gold, B) Blot probed with MAb 4D3. Cells were grown in KDM2-C (Lanes 1,4,7,10), KDM2-S (Lanes 2,5,8,11), or KDM2-NS (Lanes 3,6,9,12). The position of the 57 kDa protein is marked by an arrow. (Abbreviations: CELLS-cells remaining after distilled water extraction; SAS1/SAS2-remaining fluid from first or second SAS concentration; SADWE-Soluble antigen produced with distilled water extraction).

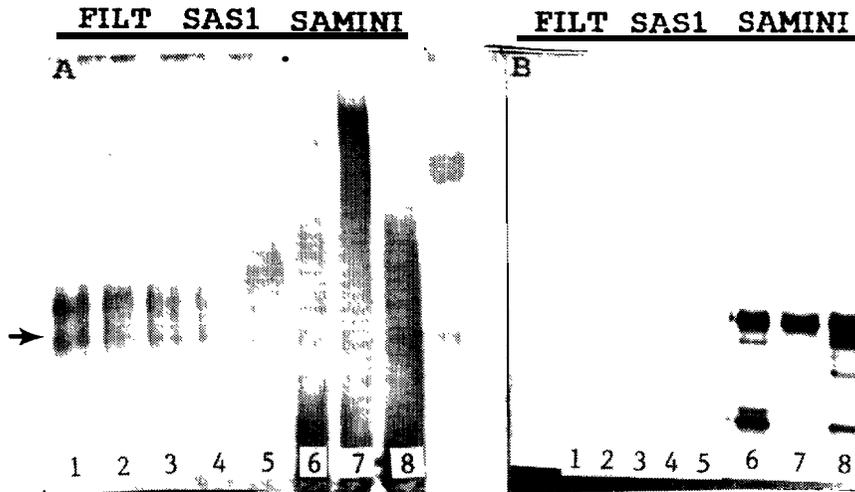


Figure 4. Western blots comparing ultrafiltration for the production of soluble antigen from ~~Renibacterium salmoninarum~~. A) Total protein stained with colloidal gold, B) Blot probed with MAb 4D3 and 3H1. Cells were grown in KDM2-C (Lanes 1,4,6), KDM2-S (Lanes 2,5,7), or KDM2-NS (Lanes 3,8). The position of the 57 kDa protein is marked by an arrow. (Abbreviations: FILT-Filtrate from ultrafiltration: SAS1-remaining fluid from first SAS concentration: SAMINI-Soluble antigen produced with ultrafiltration).

techniques similar to those used in ELISA analysis. It is a commonly used tool for the confirmation of serious human disease conditions, and it is possible that it can be adapted for the examination of fish positive by ELISA at low levels.

A traditional limitation of Western blotting in clinical and laboratory situations has been sensitivity. As described previously (Kaattari et al. 1989), it is difficult to surpass limits of 250 ng/ml of the 57 kDa protein using standard blotting procedures. However, recent advances in detection technology have increased the sensitivity of the Western blot. We explored one of these new detection methods for its application in the identification of fish infected with RS. A flourogenic peroxidase substrate was used in place of the conventional chromogenic enzyme substrate in the final step of the blotting protocol.

Two experiments were conducted to demonstrate the differences in sensitivity of the different blot development systems. First, a highly positive tissue sample was serially diluted with negative tissue. The dilutions were electrophoresed, transferred, and probed with antibodies, prior to development with the different systems. Examination of identical blots demonstrated that the chromogenic substrate allowed the detection of antigen to a concentration of 425 ng/ml (Figure 5A, lane 2), while the ECL detection system allowed detection to 2.8 ng/ml of sample (Figure 5B, lane 9). No

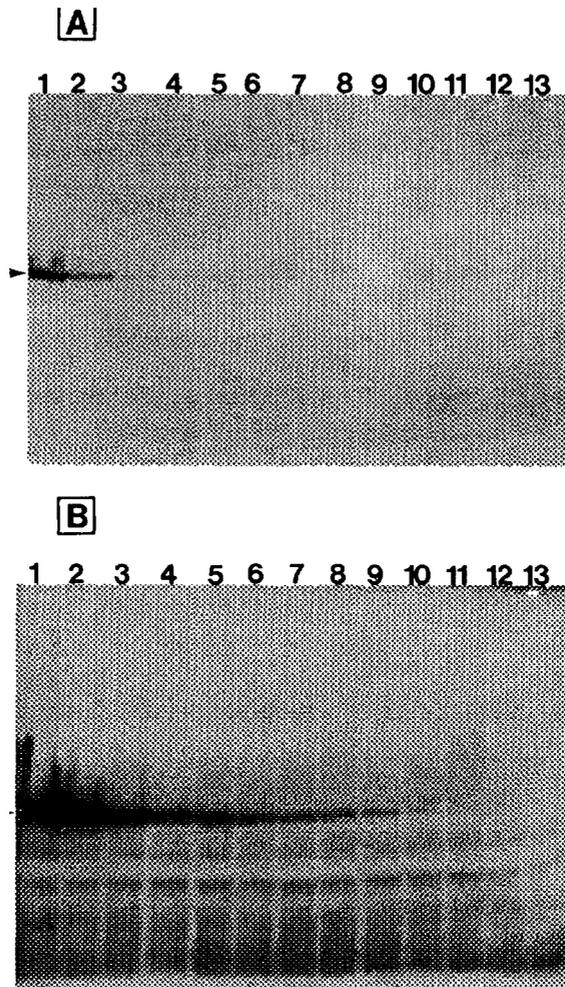


Figure 5. Comparison of substrates used in Western blot for detecting RS in kidney tissue samples. Supernatant from a high ELISA chinook salmon was diluted with supernatant from a low ELISA fish and the dilutions were subjected to electrophoresis. Identical Western blots were developed with conventional peroxidase substrate (A) and with the fluorogenic ECL substrate (B). Amounts of antigen were calculated from the monoclonal antibody-based ELISA. The position of the 57 kDa protein is marked by an arrow. Lanes: 1) 56.3 $\mu\text{g/ml}$, 2) 11.3 $\mu\text{g/ml}$, 3) 2.2 $\mu\text{g/ml}$, 4) 450 ng/ml , 5) 90 ng/ml , 6) 22.5 ng/ml , 7) 11.3 ng/ml , 8) 5.6 ng/ml , 9) 2.8 ng/ml , 10) 1.4 ng/ml , 11) 700 pg/ml , 12) 350 pg/ml , 13) negative tissue.

reactivity was observed at the 57 kDa position in the negative tissue sample used as diluent (Figure 5B, lane 13).

A second experiment examined kidney tissues from fish with concentrations of antigen ranging from 10-10,000 ng/ml (Figure 6). In this experiment, the standard development technique did not detect antigen in fish at 150 ng/ml (Figure 6A), while the ECL reagents allowed detection down to 10 ng/ml (Figure 6B). These concentrations translate into approximately 600 pg/lane and 40 pg/lane, respectively.

Other development systems were also tried in these experiments, including the use of phosphatase in an enzyme conjugate, and the use of the biotin-streptavidin system. The phosphatase system was used with conventional chromogenic substrates while the streptavidin system was used with both chromogenic and fluorogenic substrates. Each of these alternate methods resulted in extensive background problems and were therefore not considered further (data not shown).

The results demonstrate that the use of fluorogenic substrates can increase the sensitivity of Western blotting, in this application, approximately 50-100 fold over conventional peroxidase substrates. This has allowed the detection of antigen concentrations as low as 3 ng/g in kidney tissue, which is approximately the level of sensitivity of the monoclonal ELISA. One complication with the increased sensitivity, however, is the increase in background due to the use of a second antibody (goat anti-mouse peroxidase conjugate). Further experimentation will

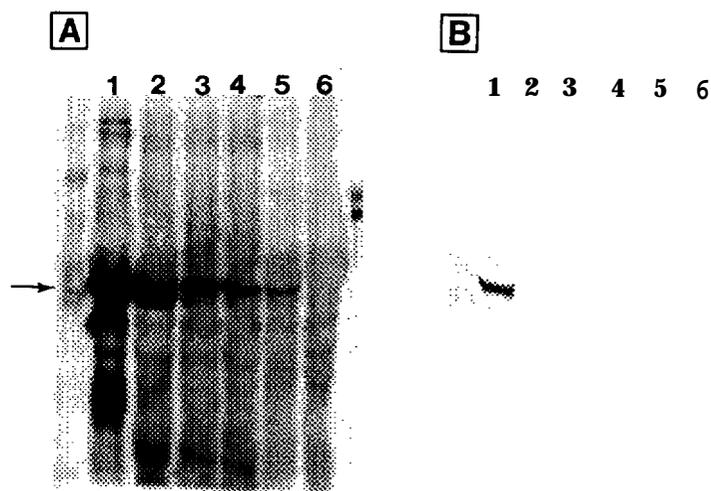


Figure 6. Examination by Western blot of kidney tissue samples from spring chinook salmon testing high and low by ELISA. Identical blots were developed with conventional peroxidase substrate (A) and with fluorogenic ECL substrate (B). The position of the 57 kDa protein is marked by an arrow. Antigen concentrations were determined with the monoclonal antibody-based ELISA. Lanes: 1) 10 $\mu\text{g/ml}$, 2) 150 ng/ml , 3) 80 ng/ml , 4) 50 ng/ml , 5) 10 ng/ml , 6) tissue below detection limit.

be performed to reduce this background by conjugation of the HRPO enzyme directly to the MAb. Additionally, we will use the technique to examine a spectrum of tissue samples in order to determine its utility in confirmation of low positive fish identified by ELISA.

Development of a Rapid, Field ELISA. In order to further simplify testing for RS in hatchery situations a rapid, field ELISA was developed which requires little training and no electronic equipment. One of the main limiting steps in the quantitative ELISA is the physical removal of the kidney sample for processing. In the field ELISA this step was simplified by the use of a cotton swab which was inserted into the kidney in a similar manner as for preparing swabs for fluorescent antibody analysis. The swab is placed in detecting reagents and color development is compared visually to a standard positive and negative tube. This assay takes approximately two hours to complete and can be performed at the spawning facility. The sensitivity of the assay was determined by incubating swabs with known concentrations of antigen, and also incubating the swabs in infected fish tissue. The sensitivity of the field ELISA approached 10 ng/ml (Figure 7) when tested with antigen used for the standard curve. To determine the sensitivity and specificity of the assay, 150 spawning chinook from the Minto facility and South Santiam Hatchery were tested by the monoclonal ELISA, FAT, and field ELISA. The correlation between the monoclonal ELISA and the field ELISA was 96%, and between the field ELISA and FAT

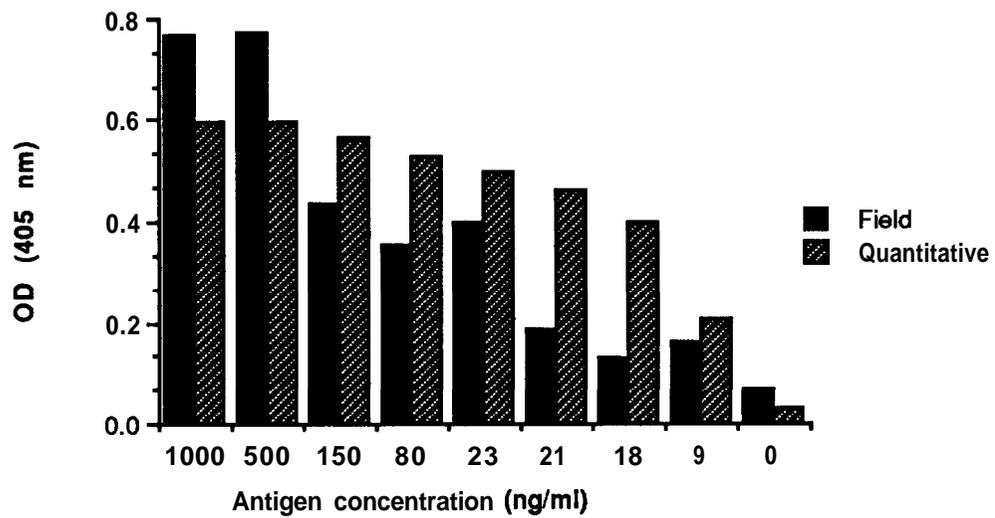


Figure 7. Comparison of detection sensitivities of the field ELISA and the monoclonal antibody-based ELISA.

94%. Disagreements between the assay systems occurred in very low positive and negative FAT samples. In the hatchery situation the field ELISA was able to detect positive fish which had 50 ng/ml levels of antigen (Table 2).

III. Testing of Samples from Naturally and Artificially Infected Fish

Quantitation of RS Antigen in Hatchery Samples. Testing of different tissues of fry for examination by polyclonal ELISA was conducted on a sample of fish taken at Carson Hatchery in February, 1989. Each of the tissue portions proved adequate for dilution and testing by ELISA. Samples of head, back, and kidney provided the most consistent results, as there appeared to be some interference from substances associated with the stomach and its contents:

	<u>TISSUE</u>					
	<u>Whole</u>	<u>Head</u>	<u>Kidney</u>	<u>Head, Back, Kidney</u>	<u>Back, Stomach</u>	<u>Stomach, Tail</u>
N	9	10	10	10	10	10
Mean OD	0.073	0.065	0.067	0.062	0.086	0.096
Std Dev	0.007	0.005	0.005	0.005	0.020	0.016
Weight (g)	1.00	0.28	0.42	0.67	0.24	0.25

Testing of yearling fish by polyclonal ELISA yielded OD readings in the range of 0.070-2.839. Fifty-seven percent of the fish tested had OD values greater than 0.100, indicating they were positive for RS antigen. These results indicate that RS antigen was present in yearling fish at Carson Hatchery at levels which were easily detectable by the polyclonal ELISA.

Table 2. Analysis of 150 adult chinook salmon by the monoclonal ELISA, field ELISA, and direct FAT.

Sample Number	Sex	Monoclonal Antigen (ng/ml)	Field ELISA (+/-)	FAT (0, 1-4+)
1	F	59945±18306	+	4+
2	M	17506±3844	+	4+
3	F	9863±2962	+	4+
4	F	4180±2074	+	1+
5	M	1130±256	+	4+
6	M	119±25	+	2+
7	M	53±23	+	2+
8	F	9±2	-	0
9	F	8±2	-	1+
10	M	5±2	-	0
11	F	4±2	-	1+
12	M	4±1	-	0
13	M	4±1	-	0
14	M	<3	-	1+
15	M	<3	-	1+
16	F	<3	-	1+
17	F	<3	-	1+
18	F	<3	-	1+
19	M	<3	-	1+
20	M	<3	-	1+
21	M	<3	+	1+
.	.	<3	-	0
.	.	<3	-	0
.	.	<3	-	0
150	...	<3	-	0

Quantitation of **RS** Antigen from Artificially Infected Fish. Since erythromycin is frequently administered to salmonid fish at some time during the spawning or rearing process, insight into the effect of the antibiotic on the ability of ELISA to detect RS antigen is important. Experiments to investigate these effects were conducted by staff at the Seattle Lab and at Oregon State University.

The chinook salmon obtained from Green River Hatchery were screened on arrival by polyclonal ELISA to establish a baseline level for comparison. This sample, and a sample of fish held at Seattle and screened at 51 days, showed 10% and 13% of the fish, respectively, positive for RS antigen (OD >0.100). Optical density levels in fish that were fed erythromycin were lower than either of the other groups injected with RS, but significantly higher than controls (Figure 8). The OD values for the group fed erythromycin were approaching the OD values of the other groups as the experiment was terminated. The culture of tissue samples indicated reduced viability in groups injected with or fed the antibiotic. No bacteria could be cultured from fish injected with medium.

When erythromycin was fed prior to RS challenge, levels of antigen were lower at 10, 30 and 40 days post challenge in the experiment performed at Oregon State University (Figure 9). Feeding of erythromycin following experimental infection had little effect on levels of antigen. Obtaining experimental fish which were not infected with bacterial kidney disease was not

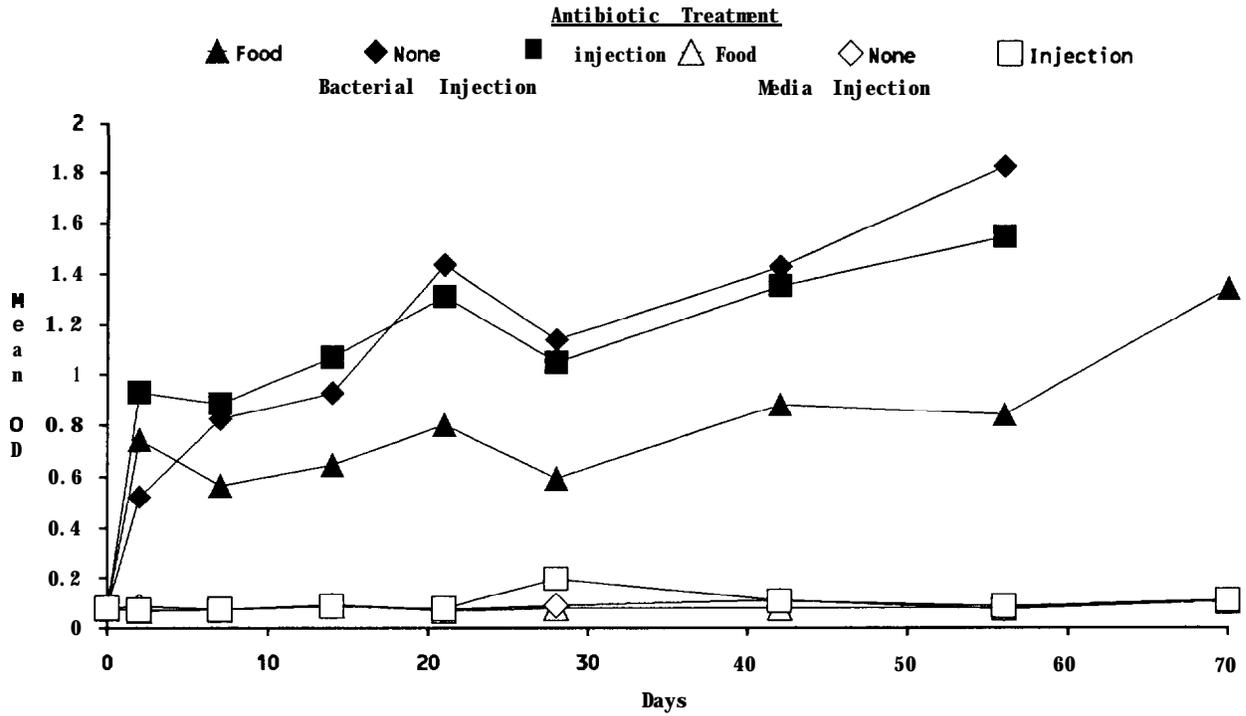


Figure 8. Distribution of mean optical density values for artificially infected chinook salmon (Green River Hatchery, RS challenge strain D6).

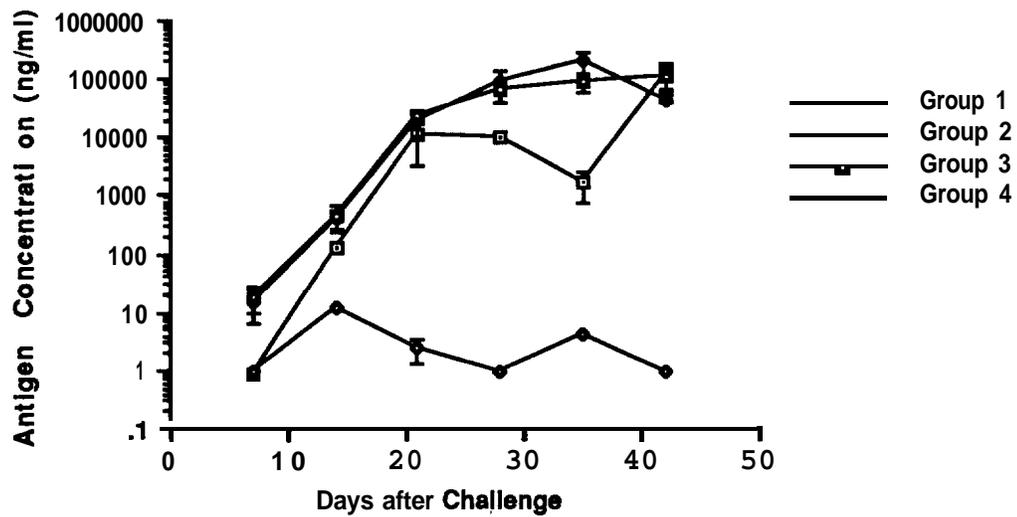


Figure 9. Effects of oral administration of erythromycin on concentrations of antigen to RS in juvenile coho salmon. Treatments are as follows: Group 1) fish fed erythromycin 14 days prior to challenge and 7 days post challenge; Group 2) fish fed erythromycin for 21 days after challenge; Group 3) fish not fed erythromycin and challenged; Group 4) fish not fed erythromycin and not challenged. Duplicate tanks were used for each group and three fish were sampled weekly from each tank. Values represent mean concentrations of antigen \pm standard error.

possible, and levels of antigen above 3 ng/ml were detected in 22% (8 of 36) of the unchallenged control fish. Additionally, fish were challenged with a high dosage of RS, which resulted in complete mortality of challenged groups in 1-2 months.

IV. Selection and Modification of Hatcheries for segregation Experiments

Installation of Individual Rearing Units. Carson Hatchery is equipped to incubate the eggs from 1200 matings in separate containers and with individual water supplies from fertilization until the eggs become eyed and are pooled into incubation stacks for hatching (about 40-45 days). To accommodate the eggs after eye-up and to raise the fry and fingerlings, eight banks of 12 or 13 tanks (99 total) were installed at the hatchery. The tanks can remain in place from December through July, before hatchery needs require the space occupied by the tanks. It is planned to rear fish in these tanks for a minimum of six months and to collect samples periodically for testing by ELISA.

At Marion Forks Hatchery 80 individual buckets for incubation of eggs were installed prior to the spawning season. Eggs from a single male-female cross are being reared in the buckets until the first feeding (approximately six months after fertilization). The four treatment groups will be randomly divided into triplicate circular tanks and later placed into individual 15 foot circular ponds.

V. Determination of **BKD** Infection of Tissue from Adult Spring Chinook Salmon for Brood Stock Segregation Distribution of ELISA Values Among Adult Spring Chinook Salmon Based on Samples of Kidney Tissue. Based on the testing of different tissue samples and fluids in the first year of this study (Kaattari et al. 1989), kidney tissue was selected at both hatcheries for testing by ELISA. Last year's results indicated problems with the use of ovarian fluid (OF) for testing by polyclonal ELISA. Examination by filtration FAT of questionable OF (OF negative by polyclonal ELISA, but positive by polyclonal ELISA with kidney tissue) showed bacteria were present in 74% (20 of 27) of the samples. Fish negative by polyclonal ELISA of kidney tissue but positive by ELISA of OF showed no bacteria by filtration FAT, indicating the presence of some type of interfering substance in the OF or of cross contamination. Testing of OF by monoclonal ELISA yielded a lower percentage of positive samples (Kaattari et al. 1989) when compared to testing of kidney tissue.

Concentrations of antigen among segregated adult fish at Marion Forks Hatchery are presented in Figure 10. Approximately 50% of the fish injected with erythromycin died prior to spawning due to undetermined causes, possibly related to handling stress. The percentage of fish with levels of antigen above 3 ng/ml was 10.8% (n=259) for males and 11.2% (n=181) for females. The fish with the highest levels of antigen were used for the crosses. Fish not injected with erythromycin and testing high by ELISA averaged 1450 ng/ml of antigen for males and 14,776 ng/ml for

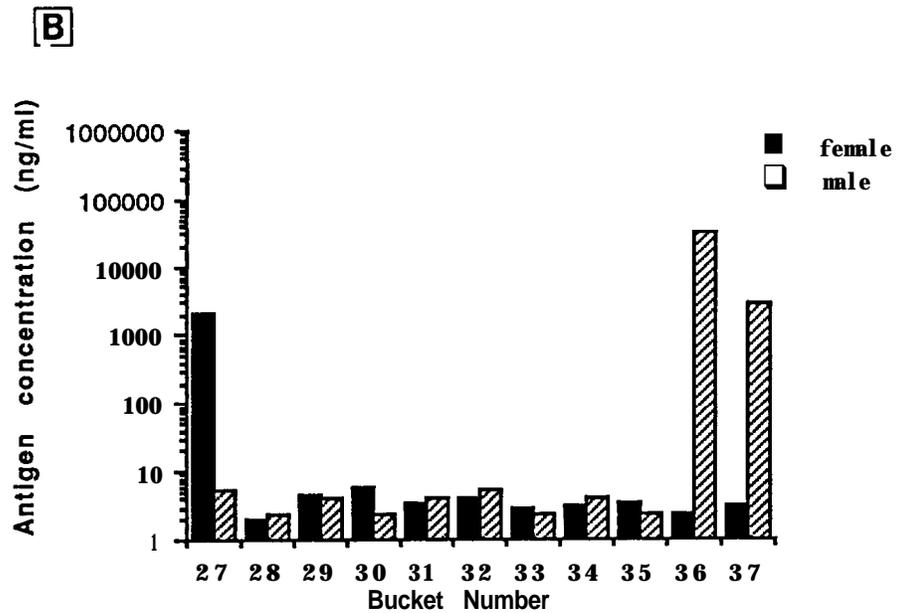
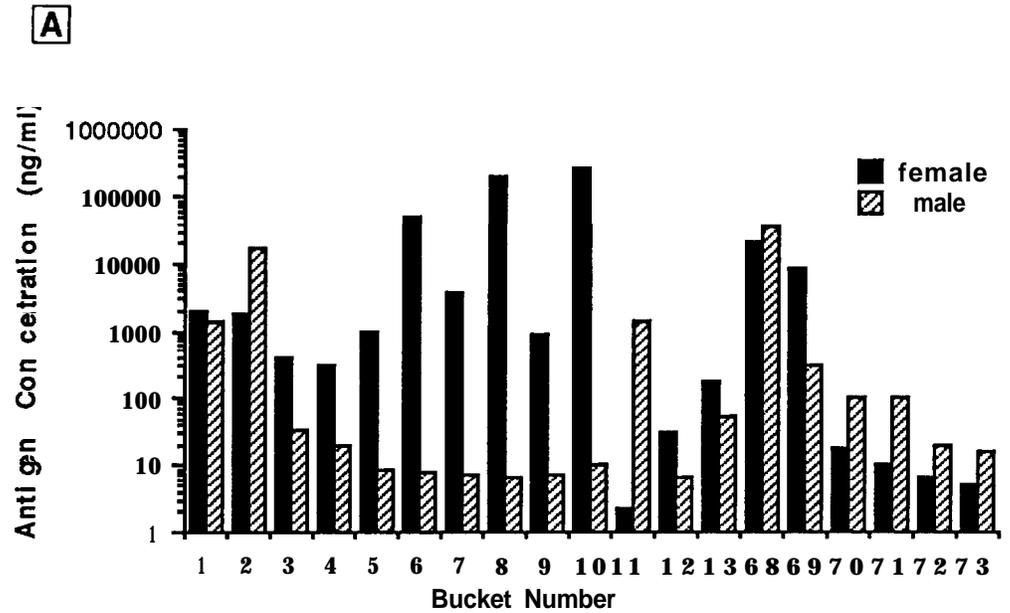


Figure 10. Levels of antigen to RS determined by the monoclonal antibody-based ELISA for adult chinook salmon selected for segregation at Marion Forks Hatchery. Gametes from each cross were incubated in individual buckets and assigned a number. A) Adults not injected with erythromycin with high levels of antigen. B) Adults injected with erythromycin with high levels of antigen.

females. Fish receiving antibiotic averaged 1583 ng/ml for males, while females averaged 101 ng/ml. Since fish return continuously and co-mingle in the river, no comparison between antigen levels and return time could be ascertained. Female fish injected with erythromycin had a lower average concentration of antigen than uninjected fish, however, it is uncertain if this is due to the erythromycin or differential mortality while in the holding pond.

The results for 1830 returning adult fish (750 male, 1080 female) at Carson Hatchery are presented in Figure 11. Fish having ELISA values below 0.100 predominated for both sexes, males 95% and females 82%. Numbers and mean OD for adult male fish are presented in Figure 12. The mean OD for fish receiving antibiotic injection(s) (INJ1, INJ2) was slightly lower than for the fish receiving no injection (UN (E,M,L)). Fish holding in the river and returning after mid-July (UN(L)) had OD values similar to fish in the other groups.

Returning adult female fish had a higher overall percentage of fish considered positive (OD > 0.100), and all of the groups had positive OD values by the end of the third week's spawning, Figure 13. The high mean OD for the females spawned on August 20 (UN (E,M)) is the result of a single extremely high individual and a low number of fish in this group. The fish holding in the river and returning after mid-July showed a consistent increase in OD values.

Selection of spawning pairs used for the segregation experiment was based wholly on ELISA results for the female fish.

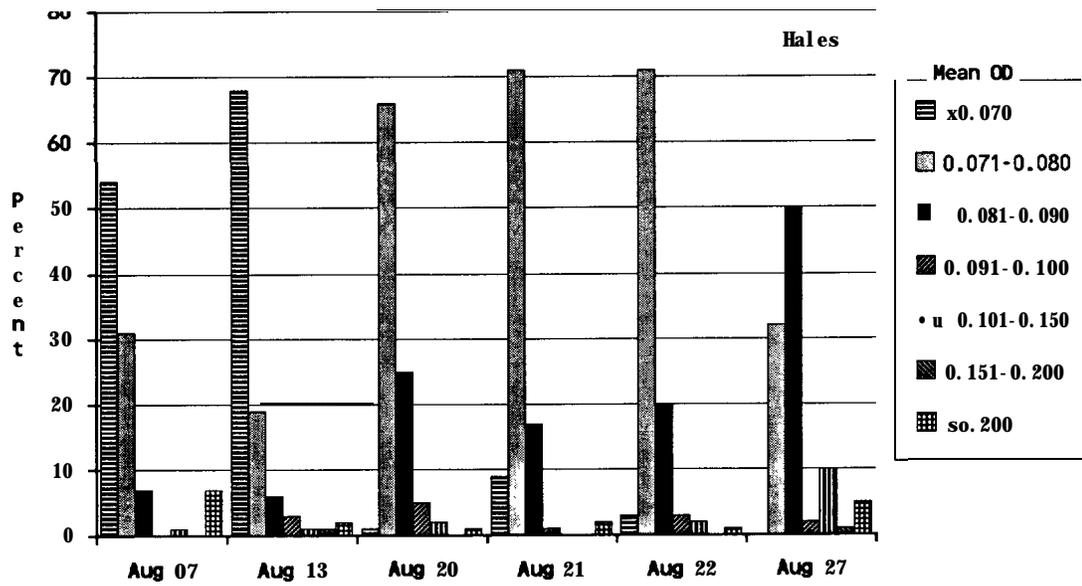
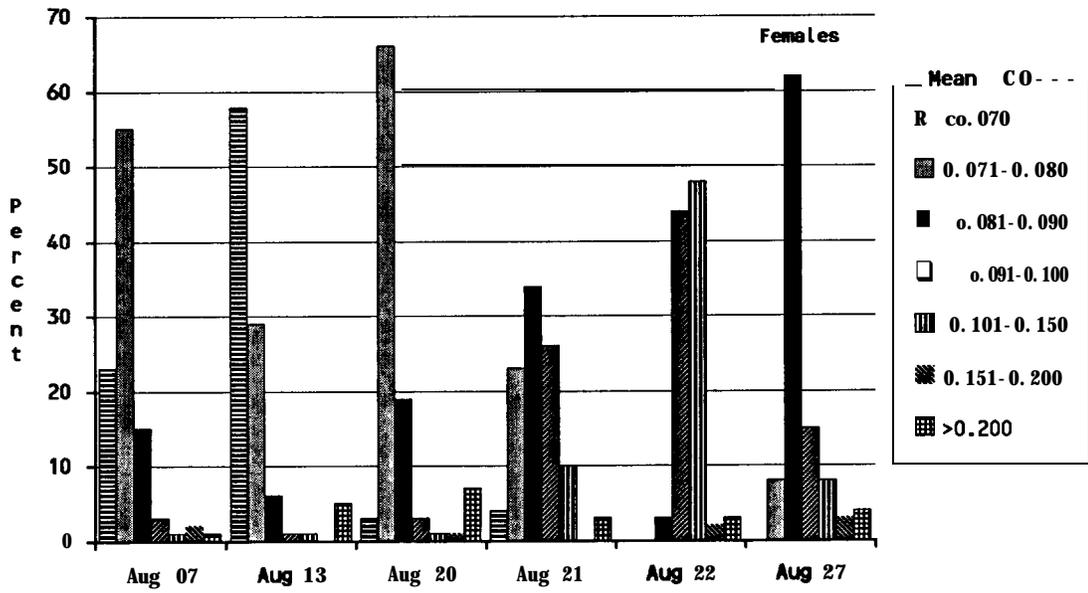


Figure 11. Percent occurrence of optical density values determined by polyclonal antibody-based ELISA for adult chinook salmon at Carson National Fish Hatchery spawned August 7-27, 1990.

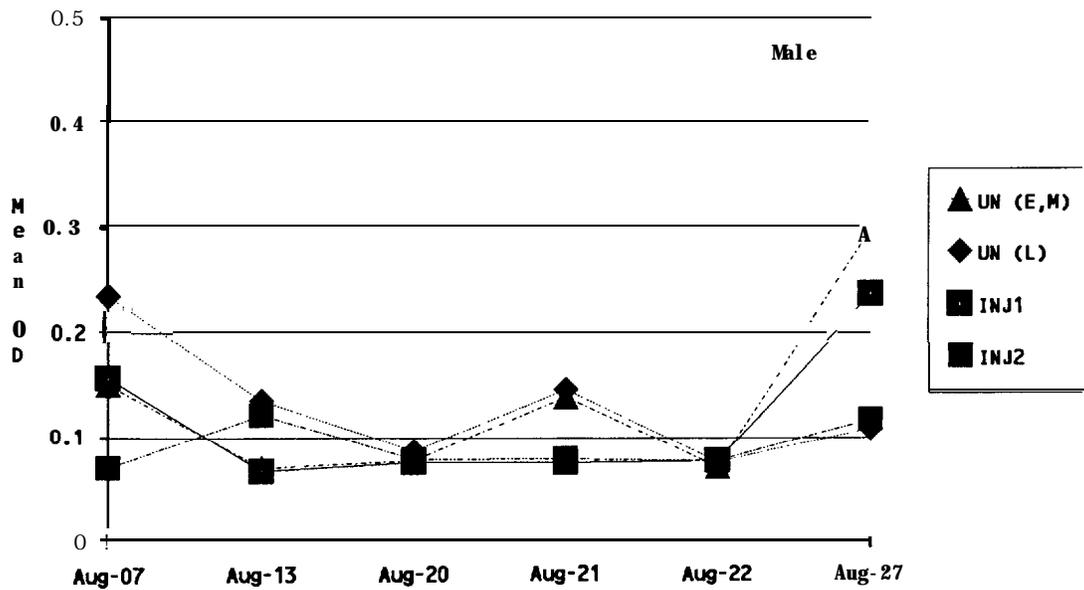
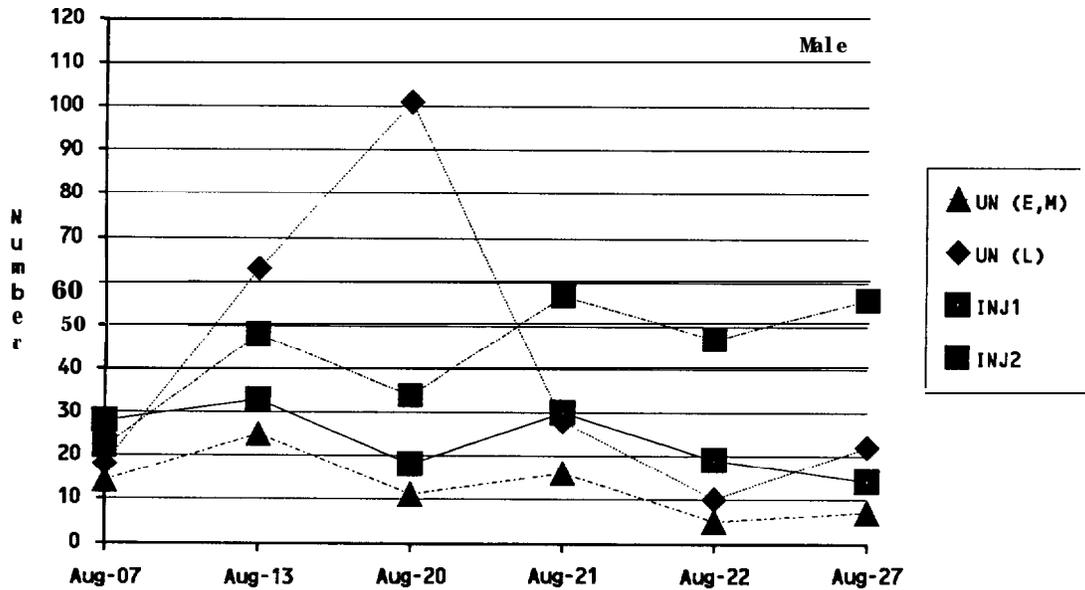


Figure 12. Number and mean optical density values determined by polyclonal antibody-based ELISA for adult male chinook salmon at Carson National Fish Hatchery, grouped by antibiotic treatment and timing of return, spawned August 7-27, 1990. (Abbreviations: UN-no injection of erythromycin; INJ1/2-injected once or twice with erythromycin; (E,M)-returning to hatchery May 10-July 11, 1990; (L)-returning to hatchery after July 11, 1990).

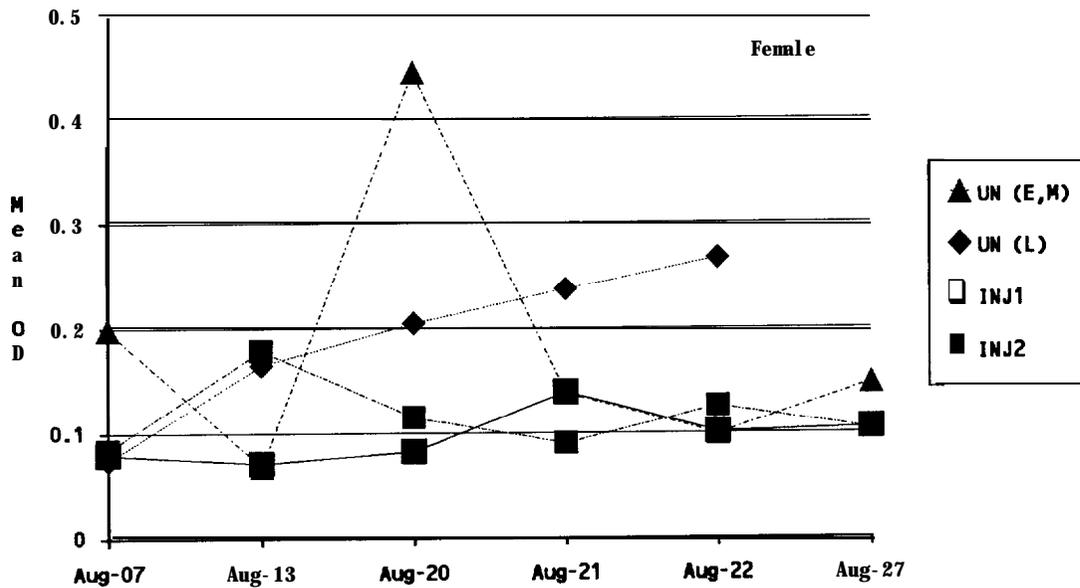
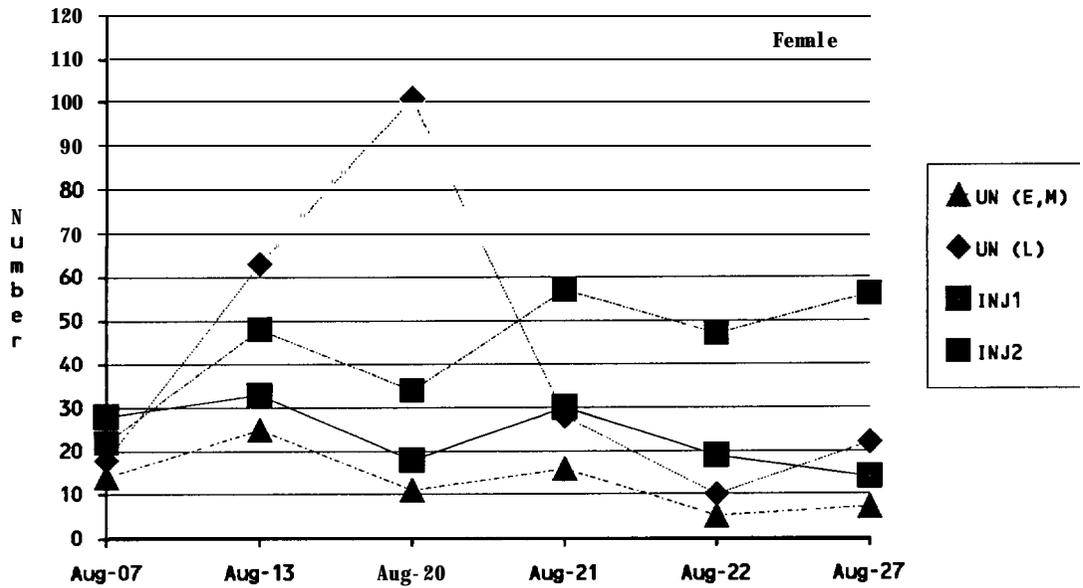


Figure 13. Number and mean optical density values determined by polyclonal antibody-based ELISA for adult female chinook salmon at Carson National Fish Hatchery, grouped by antibiotic treatment and timing of return, spawned August 7-27, 1990. (Abbreviations: UN-no injection of erythromycin; INJ1/2-injected once or twice with erythromycin; (E,M)-returning to hatchery May 10-July 11, 1990; (L)-returning to hatchery after July 11, 1990).

For the low groups, no crosses were included using males with high values and females with low values. Distribution of ELISA values for each of the treatment groups and the replicate series is presented in Figure 14. Appendix 3 shows the information on ELISA values for individual fish, timing of return, and spawning date.

VI. Determination of the Extent of Vertical Transmission
 from Adult spring Chinook Salmon to Progeny

Testing of Eggs by ELISA. Eggs from fifty-five of the individual tanks at Carson Hatchery were tested with the polyclonal ELISA. There was no significant difference in the OD values for the eggs and no correlation with the OD values from the adult female fish: (mean and range of ELISA values)

GROUP	N	ADULT		N	EGG	
		ELISA	OD		ELISA	OD
Low	22	(0.067)	-0.073-(0.081)	660	(0.100)	-0.112-(0.132)
High	33	(0.121)	-1.136-(2.701)	990	(0.094)	-0.118-(0.152)

Centrifugation prior to loading the ELISA plates was not sufficient to consolidate the yolk, oil, and embryonic tissue in the samples. Suspended components of these materials influenced the OD values, causing elevated readings as their concentration increased. The similarity of results for eggs from both low and high females and the influence of suspended material in egg samples indicates that the detection of naturally occurring antigen in eggs is not possible with the polyclonal ELISA.

Samples of eggs from Marion Forks Hatchery tested by monoclonal ELISA revealed no detectable antigen for any group. However, a uniform low level of background reaction in the egg

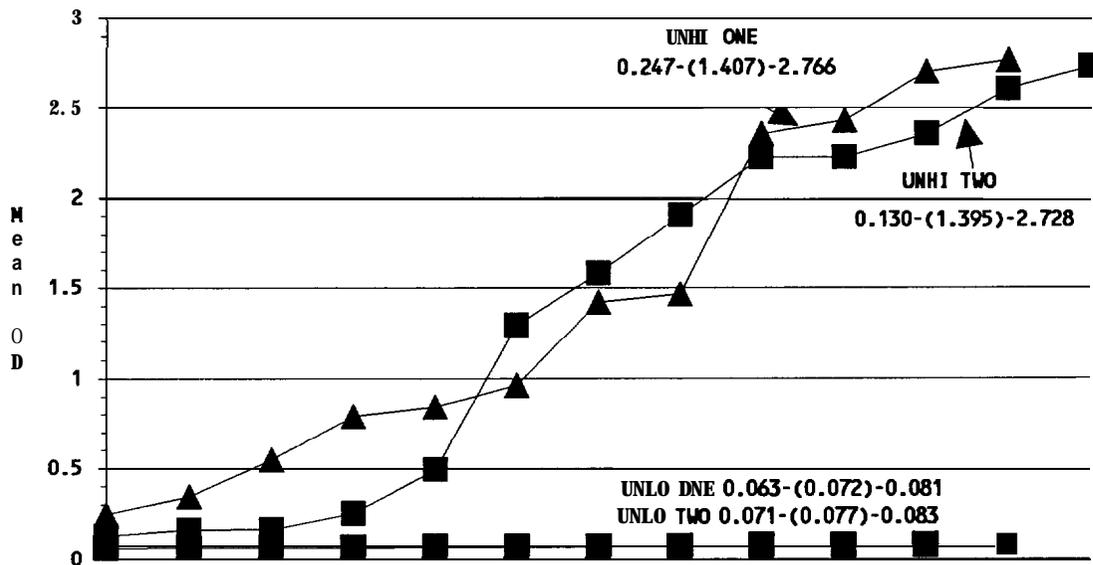
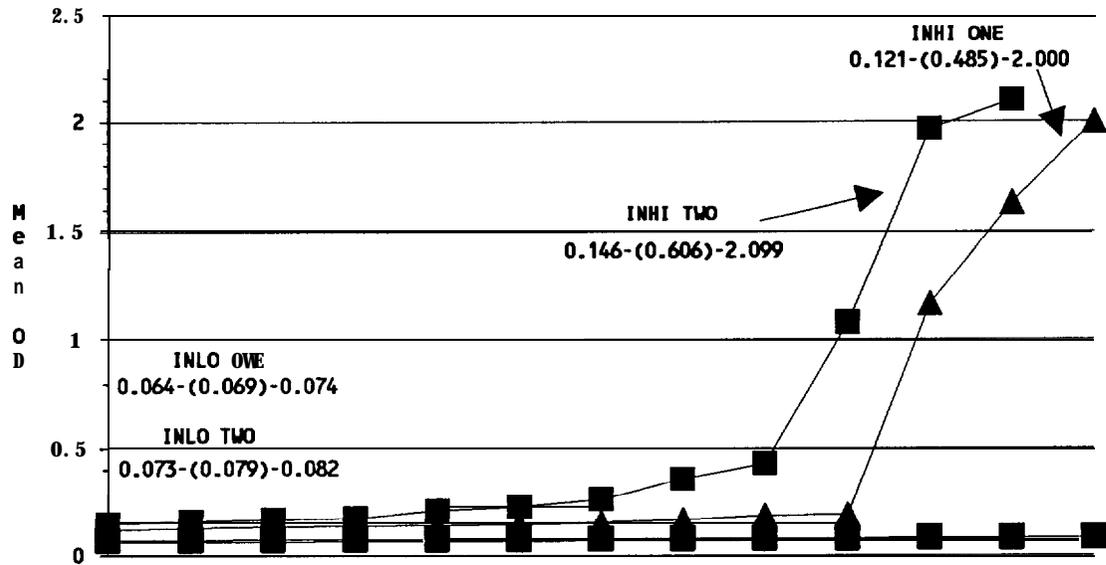


Figure 14. Distribution of mean optical density values determined by polyclonal antibody-based ELISA for adult female chinook salmon selected for segregation at Carson National Fish Hatchery, grouped by antibiotic treatment, spawned August 7-27, 1990. (Abbreviations: IN-injected with erythromycin; UN-no injection; LO-ELISA OD <0.100; HI-ELISA OD >0.100; ONE/TWO-replicate groups).

supernatant led us to incorporate a negative control into our testing regimen. The background OD observed when a negative control MAb, MOPC 21, was substituted for MAb 4D3 was the approximately the same. Optical density values for supernatants from the eggs corresponded to concentrations of antigen below 3 ng/ml and results are presented in OD units rather than as ng/ml (Appendix 4).

Since antigen was not detected in any of the samples, concern that the antigen might be sequestered by some component within the egg was addressed. Supernatants from eggs for both high and low groups were spiked with the same antigen used to produce our standard curve. No sequestering of antigen was observed as evidenced by comparison of concentrations of antigen calculated for these samples versus a simultaneously generated standard curve (Figure 15).

VII. Tagging of Bmolts and Evaluation of Returns from Experimental Groups.

Investigation of Funding. Several conferences over the course of the year with cooperating investigators and funding agencies resulted in funds being budgeted for the tagging and monitoring of the experimental fish at Carson Hatchery. An ongoing study of tagging mortality at Carson Hatchery (Project Leader, Lee Blankenship), funded by the Bonneville Power Administration, will be augmented so the fish from the segregation study can be tagged and monitored. Sources of funding for the tagging and monitoring of experimental fish at the Marion Forks Facility continue to be explored.

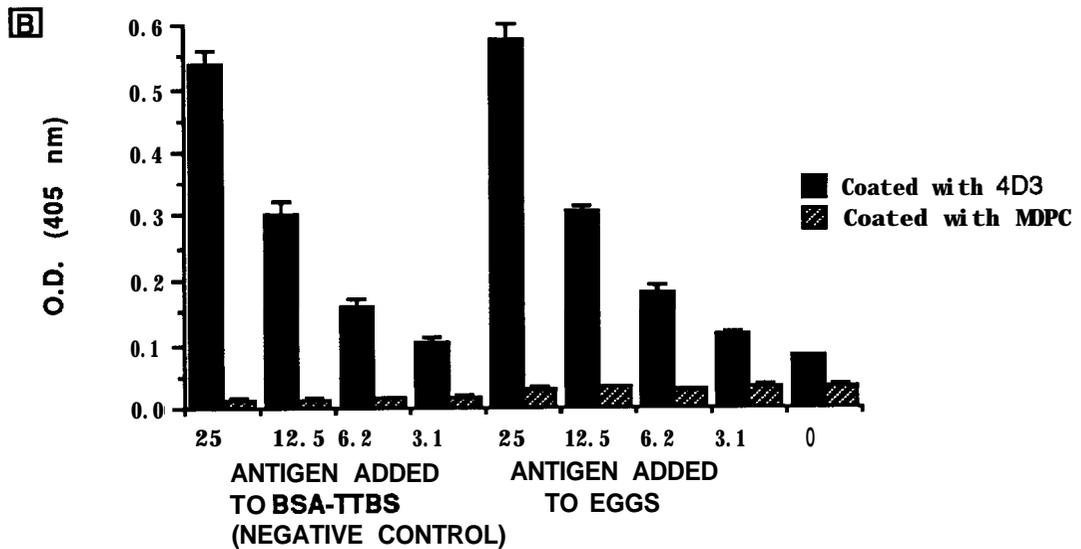
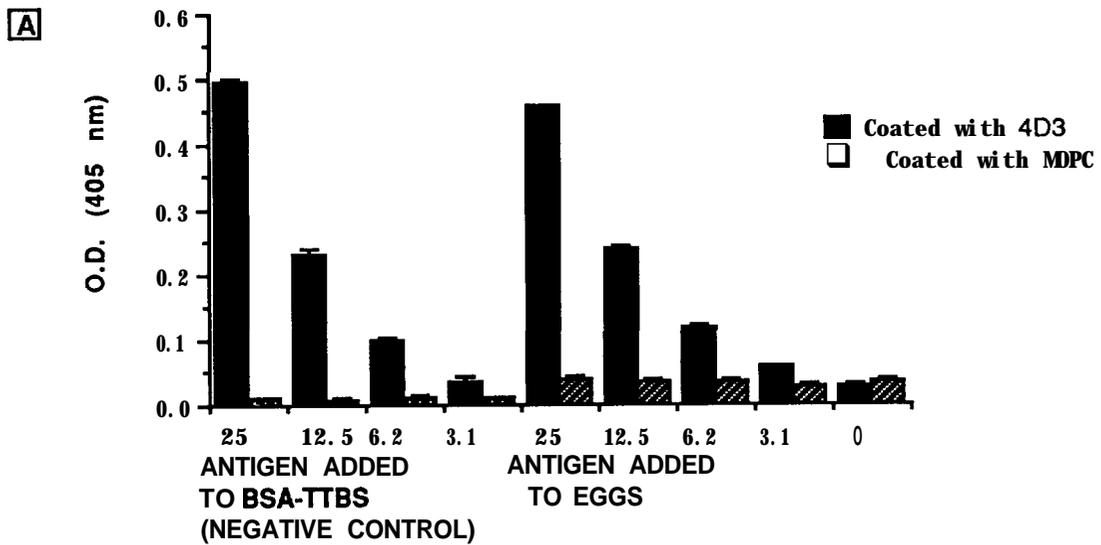


Figure 15. Assessment of nonspecific binding of supernatants from eggs for the monoclonal antibody-based ELISA. ELISA plates were coated with MAb 4D3 or MOPC 21. Dilutions of RS antigen were either made in BSA-TTBS as per standard curve or in supernatant from eggs for either adults with high levels of antigen (A), or adults with low levels of antigen (B).

LITERATURE CITED

- Banner, C.R., J.J. Long, J.L. Fryer, and J.S. Rohovec. 1986. Occurrence of salmonid fish infected with Renibacterium salmoninarum in the Pacific Ocean. *J. Fish Dis.* 9:273-275.
- Bullock, G.L. and R.L. Herman. 1988. Bacterial kidney disease of salmonid fishes caused by Renibacterium salmoninarum. U.S. Dept. Interior Fish Wildl. Serv. Fish Disease Leaflet. 78. 10 p.
- Daly, J.G. and R.M.W. Stevenson. 1985. Charcoal agar, a new growth medium for the fish disease bacterium Renibacterium salmoninarum. *Appl. Environ. Microbiol.* 50:868-871.
- _____. 1990. Characterization of the Renibacterium salmoninarum hemagglutinin. *J. Gen. Microbiol.* 136:949-953.
- Elliot, D.G., R.J. Pascho, and G.L. Bullock. 1989. Developments in the control of bacterial kidney disease of salmonid fish. *Dis. Aquat. Org.* 6:201-215.
- Evelyn, T.P.T. 1977. An improved growth medium for the kidney disease bacterium and some notes on using the medium. *Bull. Int. Epizoot.* 87:511-513.
- _____, J.E. Ketcheson, and L. Prospero-Porta. 1984. Further evidence for the presence of Renibacterium salmoninarum in salmonid eggs and for the failure of povidone-iodine to reduce the intra-ovum infection in water-hardened eggs. *J. Fish Dis.* 7:173-182.
- _____, L. Prospero-Porta, and J.L. Ketcheson. 1990. Two new technologies for obtaining consistent results when growing Renibacterium salmoninarum on KDM2 culture medium. *Dis. Aquat. Org.* 9:209-212.
- Fryer, J.L. and J.E. Sanders. 1981. Bacterial kidney disease of salmonid fish. *Ann. Rev. Microbiol.* 35:273-298.
- Harlow, E. and D. Lane. 1988. *Antibodies: a laboratory manual*. Cold Springs Harbor Lab. Cold Springs Harbor, NY. 726 p.
- Kaattari, S.L., D.D. Rockey, G.D. Wiens, L.L. Gilkey, J.R. Winton, J.L. Bartholomew, J.M. Lehner-Fournier, and R.L. Diehm. 1989. ELISA-based segregation of adult spring chinook salmon for control of bacterial kidney disease. *Ann. Rep. Contract No. DE-FG79-95906*. U.S. Dept. Energy. Bonneville Power Administration, Div. Fish Wildl. 48 p.
- Lowry, O.H., N.J. Roseborough, D.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.

Rockey, D.D., L.L. Gilkey, G.D. Wiens, and S.L. Kaattari. in press. Monoclonal antibody-based analysis of the Renibacterium salmoninarum antigen F protein in spawning chinook (Oncorhynchus tshawtscha) and coho (O. kisutch). J. Aquat. Anim. Health.

Turuga, P.S., G.D. Wiens, and S.L. Kaattari. 1987. Analysis of Renibacterium salmoninarum antigen production in situ. Fish Pathol. 22:209-214.

Wiens, G.D. and S.L. Kaattari. 1989. Monoclonal antibody analysis of common surface protein(s) of Renibacterium salmoninarum. Fish Pathol. 24:1-7.

Young, C.L. and G.B. Chapman. 1978. Ultrastructural aspects of the causative agent and renal histopathology of bacterial kidney disease in brook trout (Salvelinus fontinalis). J. Fish. Res. Board Can. 35:1234-1248.

APPENDICES

Appendix One. Monoclonal and Polyclonal Antibody-Based ELISA Protocol.

Protocol for Polyclonal Antibody-Based ELISA.

Reagent(1)	Volume (Conc)	Incubation Conditions	Incubation Time	Wash(2)
Coating Ab(3) in PBS	200 μ l (1 μ g/ml)	7°C, humid	overnight	5x
Antigen	200 μ l (???)	room temp, humid	2 hours	5x
Conjugated Ab(4) in diluent	200 μ l (0.05 μ g/ml)	room temp, humid, dark	2 hours	5x
Substrate(5)	200 μ l	35°C humid, dark	20 minutes	No
SDS Stop(6)	100 μ l 2.5%	plates read immediately following addition of stop solution		

- (1) Reagents used in the polyclonal ELISA are commercially produced by Kirkegaard and Perry Laboratories, Inc. Gaithersburg, MD
- (2) Wash Solution: 0.002M imadazole-buffered saline solution with 0.02% Tween-20 (KPL #506300)
- (3) Coating Antibody: Affinity-purified antibody to *R. salmoninarum* (GOAT)(KPL #019691) at a concentration of 1 μ g/ml in 0.01M MPB (KPL # 08400)
- (4) Conjugated Antibody: Peroxidase-labeled affinity-purified antibody to *R. salmoninarum* (GOAT) (KPL #049691) at a concentration of 0.05 μ g/ml in 0.1% non-fat dry milk borate buffer (KPL # 508201)
- (5) Substrate: Equal volumes of 2,2'-azino-di[3-ethyl-benzthiazoline sulfonate] at a concentration of 0.6 g/l in glycine buffer and 0.2% hydrogen peroxide in citric acid buffer (KPL # 506201)
- (6) SDS Stop Solution: 2.5% dodecyl sulfate in water (KPL # 508501)

Protocol for Monoclonal Antibody-Based ELISA.

Reagent	Volume (Conc)	Incubation Conditions	Incubation Time	TBBS Wash
MAb 4D3 in PBS	50 μ l (3 μ g/ml)	17°C	overnight	No
BSA in TBBS	200 μ l (0.01 g/ml)	room temp	1 hour	5x
Antigen	50 μ l (???)	17°C	2 hours	10x
MAb 3H1 in TBBS	50 μ l (1 μ g/ml)	room temp	1 hour	10X
SA-HRPO in TBBS	50 μ l (0.25 μ g/ml)	room temp	1 hour	10x
Substrate	50 μ l(1)	room temp, dark	15 minutes	No

- (1) Substrate: 0.015% hydrogen peroxide, 0.2 mg/ml ABTS
(2 2'-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid])
in 10mM citric acid buffer, pH 4.0

Appendix Two. Standard Western Blot Protocol.

Protocol used for examination by immunoblotting follow those outlined in Harlow and Lane (1988) with the following modifications. Two gels were made for each sample to be tested, allowing separate staining for total protein and antigen assessment. For the initial electrophoresis, ingredients for making a 12% resolving gel and stacking gel were utilized, excepting 3M Tris, pH 8.8, was used. Samples were boiled at 100°C for 5 min in an equal volume of sample buffer with the following formula:

Sample Buffer	
(filtered through 0.22 micron filter just prior to use)	
Trizma base	0.15 g
SDS	0.4 g
2-mercapto-ethanol	1.0 ml
Glycerol	2.0 ml
Distilled, deionized water	7.0 ml
Bromphenol Blue	0.02 g

The wet electrophoretic transfer protocol was used to transfer proteins from the two polyacrylamide gels to nitrocellulose membranes. The transfer buffer-2 was used as the molecular weight of the antigen being sought is below 80,000. Transfer was effected at 80 volts for 1.5 hours.

From each sample, one of the nitrocellulose membranes was stained to detect the total protein present in the sample. A commercially available colloidal gold stain (AuroDye forte; Jansen Biotech N.V., Olen Belgium) was employed for this procedure.

The remaining nitrocellulose membrane was washed with TBS-Tween 20 for 15 min to block non-specific binding sites on the blot. Blocked membranes were incubated with monoclonal antibodies which recognize

the 57 kDa protein for 60 min at room temperature (22°-25°C); equal volumes of undiluted culture supernatants of Mabs 4D3 and 3H1 were combined and 3.0 ml was applied to each membrane. Blots were washed with TBS-Tween 20 for 15 min. A secondary antigen, blotting grade affinity purified goat anti-mouse IgG (H & L) horseradish peroxidase-conjugated (Bio-Rad), 20 μ l conjugated antibody diluted in 4 ml TBS-Tween 20, was added and incubated at room temperature for 60 min. Blots were then washed in TBS for 15 min.

Detection was achieved by developing the blots with a substrate of chloronaphthol (Bio-Rad HRP color development reagent). The substrate was combined with methanol (15 mg substrate + 5 ml absolute methanol), added to a solution of PBS and hydrogen peroxide (25 ml PBS + 30 μ l 30% H₂O₂), and the solution added to the blot which was incubated until bands become prominent. The blots were then rinsed with distilled water and stored at 4°C.

Appendix Three. ELISA Values for Adult Female Chinook Salmon at
Carson National Fish Hatchery.

ELISA values for adult female chinook salmon used for segregation
experiment at Carson Hatchery. (Return to hatchery: 1) May 10-June
13; 2) June 14-July 11; 3) July 12-August 27).

NO INJECTION, LOW OD

Female Number	ELISA OD	Return to Hatchery	Egg Take
1	0.073	1,2	Aug-07
2	0.075	3	Aug-07
a	0.074	3	Aug-07
9	0.077	1,2	Aug-07
12	0.070	3	Aug-07
a7	0.081	3	Aug-07
94	0.073	3	Aug-13
97	0.069	3	Aug-13
107	0.072	1,2	Aug-13
135	0.063	1,2	Aug-13
140	0.067	1,2	Aug-13
165	0.066	1,2	Aug-13
Mean	0.072		
Std Dev	0.005		

NO INJECTION, LOW OD

Female Number	ELISA OD	Return to Hatchery	Egg Take
268	0.074	3	Aug-20
274	0.076	3	Aug-20
275	0.074	3	Aug-20
278	0.074	3	Aug-20
369	0.071	1,2	Aug-20
412	0.072	3	Aug-20
920	0.082	1,2	Aug-27
922	0.078	1,2	Aug-27
931	0.082	1,2	Aug-27
975	0.080	1,2	Aug-27
976	0.081	1,2	Aug-27
1022	0.083	1,2	Aug-27
Mean	0.077		
Std Dev	0.004		

A6

NO INJECTION, HIGH OD

Female Number	ELISA OD	Return to Hatchery	Egg Take
98	2.358	3	Aug-13
100	0.549	3	Aug-13
103	0.791	3	Aug-13
124	0.247	3	Aug-13
269	2.701	1,2	Aug-20
318	1.419	3	Aug-20
320	0.344	3	Aug-20
421	0.844	3	Aug-20
446	2.430	1,2	Aug-20
603	1.465	3	Aug-21
605	0.964	1,2	Aug-21
867	2.766	3	Aug-22
Mean	1.407		
Std Dev	0.933		

NO INJECTION, HIGH OD

Female Number	ELISA OD	Return to Hatchery	Egg Take
314	2.229	3	Aug-20
316	1.581	3	Aug-20
362	1.906	3	Aug-20
396	2.605	3	Aug-20
434	2.357	3	Aug-20
503	2.226	3	Aug-21
522	1.295	1,2	Aug-21
919	0.494	1,2	Aug-27
925	0.253	1,2	Aug-27
926	2.728	1,2	Aug-27
935	0.165	1,2	Aug-27
947	0.130	1,2	Aug-27
948	0.163	1,2	Aug-27
Mean	1.395		
Std Dev	1.025		

INJECTION, LOW OD

Female Number	ELISA OD	Return to Hatchery	Egg Take	Number Injections
28	0.068	1	Aug-07	2
35	0.074	1	Aug-07	2
37	0.067	1	Aug-07	2
40	0.068	1	Aug-07	2
53	0.074	1	Aug-07	2
61	0.072	1	Aug-07	2
95	0.070	1	Aug-13	2
178	0.064	1	Aug-13	2
204	0.066	1	Aug-13	2
210	0.070	1	Aug-13	2
216	0.065	1	Aug-13	2
217	0.072	1	Aug-13	2
Mean	0.069			
Std Dev	0.003			

INJECTION, LOW OD

Female Number	ELISA OD	Return to Hatchery	Egg Take	Number Injections
328	0.073	1	Aug-20	2
357	0.076	1	Aug-20	2
368	0.076	1	Aug-20	2
371	0.077	1	Aug-20	2
393	0.078	1	Aug-20	2
407	0.077	1	Aug-20	2
418	0.073	1	Aug-20	2
939	0.081	1	Aug-27	2
940	0.081	1	Aug-27	2
941	0.084	1	Aug-27	2
1063	0.082	1	Aug-27	2
1064	0.084	1	Aug-27	2
1077	0.081	1	Aug-27	2
Mean	0.079			
Std Dev	0.004			

A7

INJECTION, HIGH OD

Female Number	ELISA OD	Return to Hatchery	Egg Take	Number Injections
243	1.166	1	Aug-13	2
265	2.000	1	Aug-13	2
333	0.187	2	Aug-20	1
606	0.140	2	Aug-21	1
666	0.147	2	Aug-21	1
683	0.186	2	Aug-21	1
763	0.121	1	Aug-22	2
786	0.143	1	Aug-22	2
793	0.164	1	Aug-22	2
795	0.152	1	Aug-22	2
a35	0.138	1	Aug-22	2
a77	1.635	1	Aug-22	2
884	0.130	1	Aug-22	2
Mean	0.485			
Std Dev	0.658			

INJECTION, HIGH OD

Female Number	ELISA OD	Return to Hatchery	Egg Take	Number Injections
295	1.964	1	Aug-20	2
636	2.099	2	Aug-21	1
689	0.209	1	Aug-21	2
712	0.357	2	Aug-21	1
905	0.430	2	Aug-27	1
924	0.160	1	Aug-27	2
946	0.172	2	Aug-27	1
963	0.225	1	Aug-27	2
969	1.079	1	Aug-27	2
1005	0.167	1	Aug-27	2
1015	0.262	1	Aug-27	2
1024	0.146	1	Aug-27	2
Mean	0.606			
Std Dev	0.714			

Appendix Four. Optical Density Values for Supernatants from Eggs at Marion Forks Hatchery.

OD values of supernatants from samples of eggs taken at Marion Forks Hatchery. OD values are an average \pm standard deviation of 20 eggs from each 1 x 1 adult cross. Eggs were assayed using either MAb 4D3 or MOPC 21.

No erythromycin injection, high concentration of antigen.

Bucket No.	Monoclonal ELISA			Control ELISA		
	Mean OD		Std Dev	Mean OD		Std Dev
1	0.049	\pm	0.007	Not Tested		
2	0.039	\pm	0.020	0.033	\pm	0.014
3	0.029	\pm	0.009	0.032	\pm	0.011
4	0.031	\pm	0.009	0.033	\pm	0.008
5	0.036	\pm	0.010	0.041	\pm	0.012
6	0.043	\pm	0.010	0.053	\pm	0.015
8	0.039	\pm	0.009	0.036	\pm	0.010
9	0.036	\pm	0.013	0.036	\pm	0.012
10	0.042	\pm	0.009	0.045	\pm	0.008
12	0.044	\pm	0.011	0.044	\pm	0.021
13	0.035	\pm	0.009	0.044	\pm	0.021
68	0.044	\pm	0.010	0.044	\pm	0.018
69	0.040	\pm	0.009	0.037	\pm	0.007
70	0.038	\pm	0.011	0.032	\pm	0.009
71	0.034	\pm	0.008	0.034	\pm	0.009
72	0.030	\pm	0.006	0.030	\pm	0.010
73	0.038	\pm	0.009	0.032	\pm	0.008

No erythromycin injection, low concentration of antigen.

Bucket No.	Monoclonal ELISA			Control ELISA		
	Mean OD	±	Std Dev	Mean OD	±	Std Dev
14	0.035	±	0.009	0.035	±	0.021
15	0.032	±	0.011	0.030	±	0.010
16	0.054	±	0.008	Not Tested		
17	0.030	±	0.012	0.030	±	0.011
18	0.038	±	0.011	0.032	±	0.009
19	0.047	±	0.011	0.042	±	0.013
20	0.036	±	0.012	0.036	±	0.011
21	0.032	±	0.009	0.029	±	0.009
22	0.043	±	0.008	0.042	±	0.006
23	0.043	±	0.008	0.042	±	0.006
24	0.056	±	0.011	0.048	±	0.009
25	0.044	±	0.011	0.044	±	0.021
26	0.039	±	0.010	0.040	±	0.012
61	0.033	±	0.009	0.032	±	0.007
62	0.038	±	0.012	0.046	±	0.017
63	0.031	±	0.004	0.046	±	0.008
64	0.035	±	0.009	0.038	±	0.007
65	0.027	±	0.006	0.038	±	0.010
66	0.027	±	0.006	0.025	±	0.007
67	0.035	±	0.008	0.036	±	0.008

Injected with erythromycin, high concentration of antigen.

Bucket No.	Monoclonal ELISA			Control ELISA		
	Mean OD		Std Dev	Mean OD		Std Dev
27	0.032	±	0.006	0.036	±	0.007
28	0.035	±	0.008	0.042	±	0.009
29	0.062	±	0.024			Not Tested
30	0.041	±	0.006			Not Tested
31	0.046	±	0.005			Not Tested
32	0.047	±	0.007			Not Tested
33	0.036	±	0.009			Not Tested
34	0.043	±	0.012			Not Tested
35	0.030	±	0.006			Not Tested
36	0.033	±	0.005			Not Tested
37	0.033	±	0.006			Not Tested

Injected with erythromycin, low concentration of antigen.

Bucket No.	Monoclonal ELISA			Control ELISA		
	Mean OD	±	Std Dev	Mean OD	±	Std Dev
41	0.035	±	0.006	Not Tested		
42	0.034	±	0.009	Not Tested		
43	0.029	±	0.005	Not Tested		
44	0.019	±	0.021	Not Tested		
45	0.023	±	0.021	Not Tested		
46	0.023	±	0.004	Not Tested		
47	0.024	±	0.004	Not Tested		
48	0.020	±	0.004	Not Tested		
49	0.018	±	0.005	Not Tested		
51	0.036	±	0.007	Not Tested		
52	0.033	±	0.006	Not Tested		
53	0.037	±	0.009	Not Tested		
54	0.040	±	0.012	Not Tested		
55	0.042	±	0.003	Not Tested		
56	0.044	±	0.005	Not Tested		
57	0.039	±	0.006	Not Tested		
58	0.041	±	0.005	Not Tested		.
59	0.039	±	0.010	0.032	±	0.007
60	0.037	±	0.009	0.034	±	0.009

All

Appendix Five. Publications and Presentations.

PUBLICATIONS

Rockey, D.D., L.L. Gilkey, G.D. Wiens, and S.L. Kaattari. in press. Monoclonal antibody-based analysis of the Renibacterium salmoninarum antigen F protein in spawning chinook (Oncorhynchus tshawytscha) and coho (O. kisutch). J. Aquat. Anim. Health.

_____, P.S.D. Turaga, G.D. Wiens, B.A. Cook, and S.L. Kaattari. in press. Characterization of a Renibacterium salmoninarum serine proteinase which digests a major autologous extracellular and cell-associated protein. Can. J. Microbiol.

Wiens, G.D. and S.L. Kaattari. in press. Monoclonal antibody characterization of a leukoagglutinin produced by Renibacterium salmoninarum. Infect. Imm.

PRESENTATIONS

Rockey, D.D., L.L. Gilkey, G.D. Wiens, and S.L. Kaattari. Use of monoclonal antibodies in the detection and epidemiological analysis of Renibacterium salmoninarum in adult chinook and coho salmon. Ann. Meet., Oregon Chapt., Amer. Fish. Soc., Timberline, OR, February, 1990.

_____. Use of monoclonal antibodies in the detection of Renibacterium salmoninarum in spawning adult Pacific salmon. Ann. Meet., Fish Health Sect., Amer. Fish. Soc., Minneapolis, MN, July, 1990.

Wiens, G.D. and S.L. Kaattari. Characterization with monoclonal antibodies of a 57 kDa salmonid leukoagglutinin produced by Renibacterium salmoninarum. Ann. Meet., Amer. Soc. Microbiol., Anaheim, CA, May, 1990.

_____. Characterization with monoclonal antibodies of a 57 kDa salmonid leukoagglutinin produced by Renibacterium salmoninarum. Ann. Meet., Fish Health Sect., Amer. Fish. Soc., Minneapolis, MN, July, 1990.