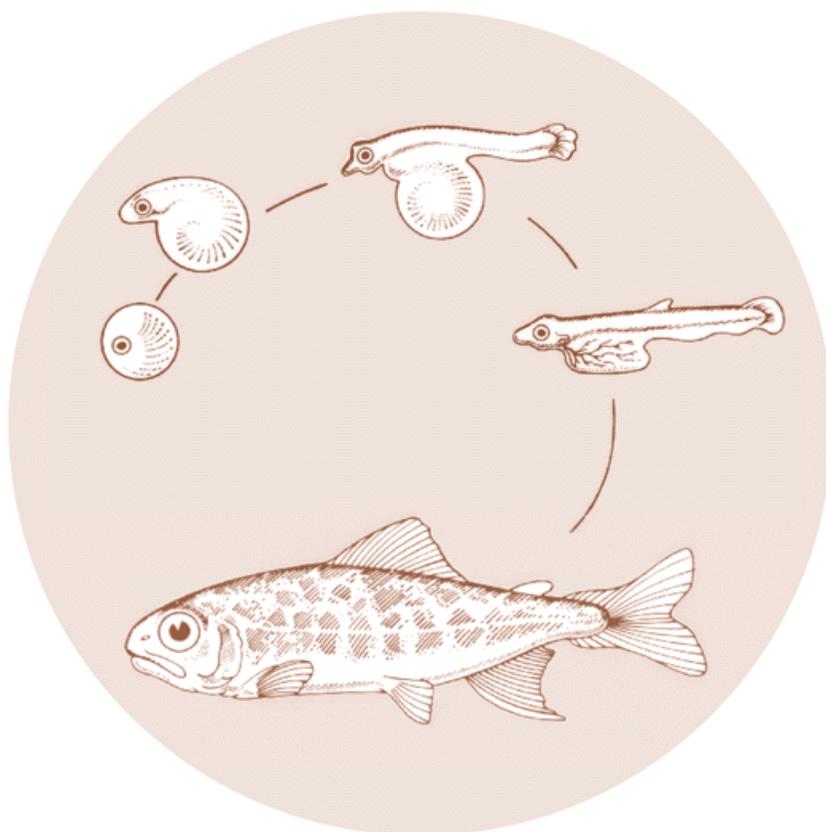


December 1989

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

Annual Report 1989



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ABSTRACT

Bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum*, 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 *R. salmoninarum* can be transmitted from parent to progeny, and therefore culling of gametes from infected parents should obviate this mode of transmission. This report presents the results from the first year of our four year study to investigate segregation of broodstock as a tool for controlling BKD. The segregations will use Enzyme-Linked Immunosorbent Assays (ELISAs) as detection systems to identify, in tissues of infected fish, proteins produced by *R. salmoninarum*. A first step in the development of the described detection systems was the optimization of the production of important antigenic proteins from *R. salmoninarum*. Different culture media were qualitatively and quantitatively evaluated for their ability to support production of cellular and soluble proteins. The major factor affecting antigen quality was the presence and absence of calf serum. Media components and *R. salmoninarum* growth products could not be separated during harvest of proteins from the cultures containing serum. This caused problems with the quantitation of actual bacterial proteins in the preparation. Thus media without serum is currently employed.

Two independent ELISA techniques for the identification of infected parents were examined. One technique is based on polyclonal antisera produced in rabbits and the second is based on mouse monoclonal antibodies (Mabs). To develop the latter system, several Mabs against a major *R. salmoninarum* antigenic protein were produced. These Mabs were used for the detection of *R. salmoninarum* antigens in infected fish and also to characterize proteins produced by the bacterium. Both ELISAs were deemed suitable for the segregation of parents into the high and low BKD groups required for this study. An alternate system for the detection of *R. salmoninarum* proteins, the Western blot, was also investigated, This technique was 100 to 1000 fold less sensitive than either ELISA system and therefore will be useful only for confirmation of highly positive tissues. Future work will attempt to increase the sensitivity of the Western blotting system. Finally, two hatcheries were identified for use in the described segregation experiments. The Carson National Fish Hatchery (Skamania County, WA) and the Marion Forks Fish Hatchery (Linn County, OR) will be used for the experiments.

INTRODUCTION

Bacterial kidney disease (BKD) is a chronic, systemic condition caused by the fastidious gram positive bacterium *Renibacterium salmoninarum*. The disease is responsible for major losses of salmon and trout from hatchery populations in the Pacific Northwest and around the world (Bullock and Herman 1988, Fryer and Sanders 1981). Salmonids are susceptible to the disease as both juveniles and adults in both freshwater and marine environments (Banner et al. 1986). Because vaccination procedures have not been shown to be effective, control of the disease is possible only through antibiotic therapy, principally erythromycin treatment, and other management strategies (Elliott et al. 1989). Control is complicated by survival of *R. salmoninarum* within host cells and within unfertilized and fertilized eggs (Young and Chapman 1978; Evelyn et al. 1984). These survival mechanisms are thought to assist in bacterial persistence and vertical transmission, respectively.

This report describes the results of the work from the first year of our four year BPA study to investigate the practice of segregation of broodstock as a tool for controlling BKD in the hatchery environment. A schematic of the experimental design is presented in Figure 1. Briefly, the project is designed to identify and segregate gametes from *R. salmoninarum* infected and uninfected spring chinook salmon (*Oncorhynchus tshawytscha*). Enzyme-linked immunosorbent assays (ELISAs) will be used to identify positive and negative adults. Gametes from parents with high antigen concentrations will be crossed with gametes from other highly positive parents, and gametes from parents **with** undetectable antigen concentrations will be crossed with gametes from similar parents. Additionally, progeny from erythromycin-injected and uninjected adults will be examined to investigate the effect of antibiotic therapy on the the ability of the ELISA-based segregation to predict bacterial levels that may be transmitted to the progeny. Each group resulting from the segregations will be reared separately and the presence of antigen and clinical disease will be monitored throughout their development. Finally, smolts will be tagged prior to release for comparison of adult returns from the different crosses. We anticipate that this project will determine the overall feasibility of the large scale use of segregation for the control of BKD in production facilities.

The ELISA systems described in this report use specific antibodies to detect *R. salmoninarum* antigens in tissues of infected fish. Soluble proteins produced by *R. salmoninarum* during infection have been used for many years as dependable diagnostic markers for the presence of the bacteria in diseased fish (Chen et al. 1974; Bullock et al., 1980). The principal antigens produced by the bacterium are a 57 kilodalton (kdal) protein and its breakdown products (Wiens and Kaattari 1989; Rockey et al. manuscript in preparation). The 57 kdal protein, labelled antigen F or hemagglutinin by different investigators (Getchell et al. 1985; Daly and Stevenson 1987), has been associated with in vitro immunosuppression (Turaga et al. 1987b) and cellular agglutination (Daly and Stevenson 1987, 1989). Therefore, in addition to its use as a diagnostic marker, antigen F may serve a significant role in the pathogenic process.

This research is a joint project between the laboratories of Dr. S. L. Kaattari of the Department of Microbiology at Oregon State University and the laboratory of Dr. J. R. Winton at the National Fisheries Research Center in Seattle, WA. This arrangement will facilitate replication of the segregations at different hatcheries. This is also useful because of different areas of expertise and different experimental approaches of the two groups.

During this first year of study our research efforts concentrated on four primary objectives. First, antigens produced by *R. salmoninarum* were examined and rabbit and mouse antibodies against these antigens were characterized. Second, the use of polyclonal antisera and monoclonal antibodies in the detection of *R. salmoninarum* antigens was investigated. Third, *R. salmoninarum* antigen concentrations in fish from selected hatcheries was investigated. Finally, hatcheries in the Columbia River system (Figure 2) suitable for the segregation experiments were identified. This report describes the progress of these four objectives and presents results which examine the application of the ELISA systems to the definitive diagnosis of BKD in suspect fish.

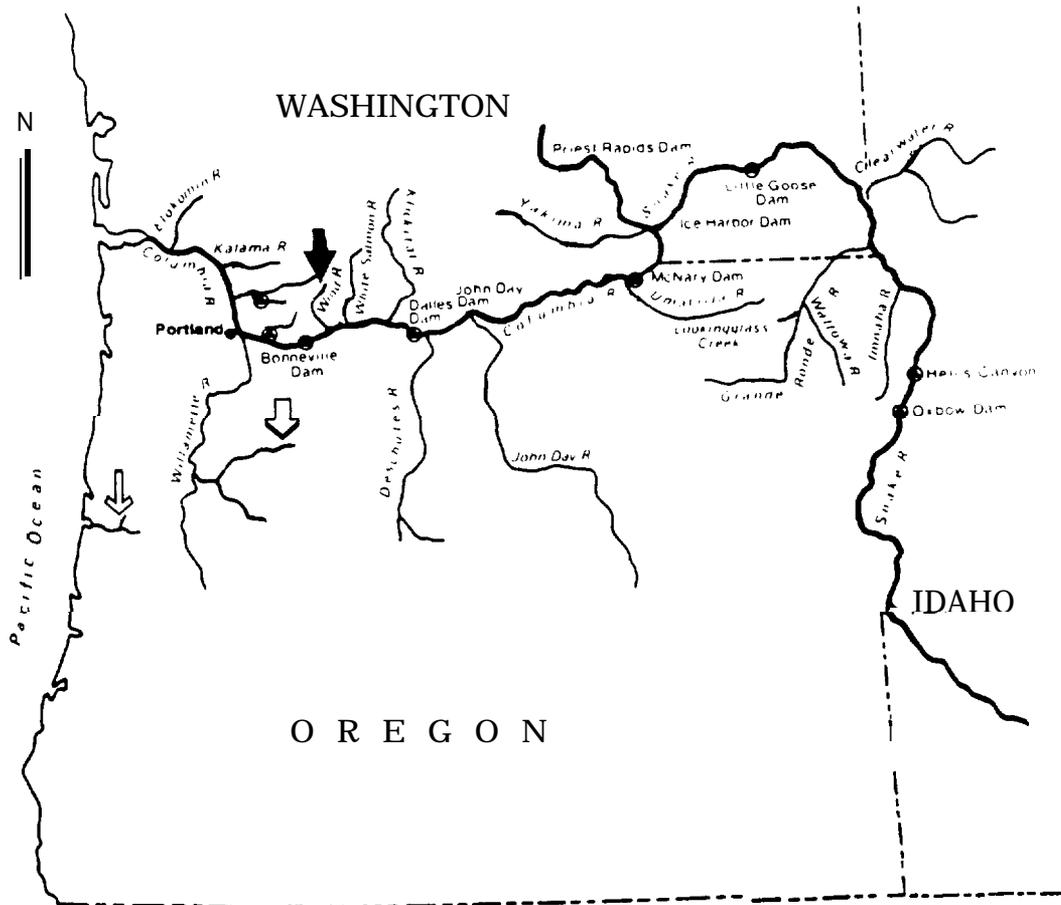


Figure 2. Map of Oregon and Southwest Washington indicating hatcheries used for sample collection. Legend: \blacktriangledown : Carson National Fish Hatchery. \downarrow : Marion Forks Hatchery and the Minto Collection Facility. \downarrow : Fall Creek Salmon Hatchery.

MATERIALS AND METHODS

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

Standardization and Analysis of Antigen Preparation.

Production of antigen by *Renibacterium salmoninarum*, ATCC strain 33209 and isolate D6 (from coho salmon, *O. kisutch*, in Oregon), was examined in both laboratories. These strains were chosen as common reference strains and are pathogenic to chinook and coho salmon. Because purified *R. salmoninarum* antigen is used to construct a standard curve for quantitation of antigen in the ELISA system, the ability to produce a consistent well characterized antigen is essential. The antigen production method must yield consistent results, be economical and the technology should be easily transferable to other laboratories.

To determine the effects of media composition on antigen production, *R. salmoninarum* was grown in six commonly used culture media: KDM-1 (10% calf serum instead of 20% human blood; Ordal and Earp 1956), KDM-2, KDM-2 plus serum (KDM-2 S; Evelyn 1977), KDM-2 plus charcoal (KDM-2 C; Daly and Stevenson 1985), ultrafiltered KDM-2 (KDM-2 UF; Turaga et al 1987b) and biphasic medium (200 ml peptone-saline broth with a KDM-2 C agar base; Daly and Stevenson 1985). Bacteria from a 10 d starter culture were used to inoculate 500 ml flasks of each liquid medium and a flask of biphasic medium. Cultures were incubated at 15°C with gentle shaking until harvest at 20 d. At harvest, cells were separated from the culture supernatant by centrifugation at 5000 x g for 30 min. Cell pellets were washed 3X in phosphate buffered saline (10 mM PO_4 , 0.85% NaCl pH 7.2; PBS), wet weights of the pellets taken and samples removed for electrophoresis. Soluble proteins were prepared from the supernatant by centrifugation at 10,000 x g for 10 min, concentration by 50% saturated ammonium sulfate (SAS) precipitation, and dialysis against three changes of PBS. The amount of total protein was quantified by the Lowry method (Lowry et al. 1951). Uninoculated media were prepared in the same manner to determine pre-inoculation protein concentrations and serve as negative controls. Dilutions of these preparations were used for ELISA and Western blot analysis of *R. salmoninarum* antigens.

The cell wash method for preparation of *R. salmoninarum* cell-associated antigen as described by Daly and Stevenson (1987) was also investigated. *Renibacterium salmoninarum* strain 33209 was grown in selected media for 10 d. Pelleted cells were washed three times in PBS and once in distilled water. The water extract was filter-sterilized (0.45 μ) and a sample was examined by Western blot analysis.

Growth studies of *R. salmoninarum* were conducted to determine the optimal harvest time for obtaining large amounts of a high quality antigen. Because results from the media comparison showed that presence or absence of serum in the media was a major factor effecting bacterial growth and antigen quality, one medium from each category was chosen for the growth study. *Renibacterium salmoninarum* strain D6 was grown in a starter culture flask of KDM-2 and 5 ml aliquots were used to inoculate 500 ml each of KDM-2 and KDM-2 S. Cultures were incubated as described above and were sampled every day for the first eight days and then on alternate days until day 20. Samples were prepared from these cultures for ELISA and Western blot analysis. These methods allowed for the qualitative and quantitative analysis of antigen production. Additionally, growth curves were conducted to relate bacterial growth with antigen production. The types and amounts of samples taken were as follows: ELISA sample - one ml culture was collected and stored at -70°C; sample for growth curve - one ml culture was serially diluted (three dilutions per sample) in PBS and 100 μ l of each dilution was spread on each of three replicate charcoal agar plates (KDM-2 C plus 1.5% agar) for a total of 12 plates per sample; Western blot sample (bacteria and supernatant) - 10 ml of the culture was removed and centrifuged to pellet the bacteria, and the pellet and supernatant were processed separately. Gram stains and plating for contaminants were done every other day.

Standardization and Analysis of Polyclonal Antisera for use in the ELISA. To determine the optimal method for producing a polyclonal antibody for use in an ELISA system the Seattle laboratory compared different production lot of polyclonal antisera that were already available for use in BKD detection. Ten different lots of polyclonal anti-*R. salmoninarum*, eight from rabbit and two from goat, were obtained from state, university and private laboratories. These antisera were produced against either whole cells or the soluble fraction by a variety of immunization protocols. This collection of polyclonal antibodies was examined for reactivity

to BKD soluble and cellular antigens by Western blot analysis. Western blots were also performed to investigate the cross-reactivity of polyclonal antisera with 10 other bacterial species. The panel of bacteria chosen for screening included the fish pathogens *Aeromonas salmonicida*, *Yersinia ruckeri* and *Vibrio anguillarum*. Also included was *Rothia dentocariosa* which has been reported to cross-react with some lots of anti-*R. salmoninarum* antisera (Dixon 1986). All bacteria used for screening were grown on KDM-2 either at 18°C or 37°C (*R. dentocariosa*), scraped from the agar and suspended in PBS to an OD of 0.01 (A545). The suspension was mixed 1:1 with sample buffer (0.15 g tris base, 0.4 g SDS, 1 ml 2-mercaptoethanol, 7 ml water, 0.002 g bromphenol blue) and 10 ml was loaded per lane for electrophoresis and transfer.

Comparison and Production of Monoclonal Antibodies (Mabs).

A major focus of the first year of the project at the Corvallis laboratory was the comparison and production of Mabs to soluble antigen. This was the first step in the development of the Mab-dependent quantitative ELISA and Western blot techniques.

Hybridoma cell lines (Balb/C mice origin) producing Mabs against soluble antigen were produced and screened using standard techniques (Wiens and Kaattari 1989, Campbell 1984). Supernatants from hybridomas produced by other laboratories were also obtained for comparison (Newman et al. 1989; Sommer et al., 1989). The collection of Mabs was first qualitatively examined for antigen recognition by Western blot using concentrated culture supernatant from *R. salmoninarum* grown in KDM-2 + C. One to five micrograms of antigen was electrophoresed on denaturing 12% polyacrylamide gels using methods described by Schleif and Wensink (1981). After electrophoresis, proteins were transferred to nitrocellulose for analysis by Western blot and colloidal gold total protein stain. All electrophoresis and blotting were conducted on a Bio-Rad Mini-Protean II electrophoresis system (Bio-Rad Laboratories, Richmond, CA) using instructions provided by the manufacturer. The Western blot technique will be detailed in Methods and Materials section III.

After their production, Mabs which reacted strongly with soluble antigen were then characterized on the basis of their suitability for use in the ELISA. For these comparisons, ascites fluid was harvested from mice which had been injected with a specific hybridoma (Campbell 1984). Antibodies were concentrated from ascites using a 50% (SAS) precipitation. The pellet

from this concentration was dissolved in and dialyzed against PBS in preparation for purification using staphylococcal protein A column chromatography. A commercial kit for purification of IgG Mabs (MAPS system; Bio-Rad Laboratories) was used according to instructions provided. After purification, antibodies were again concentrated with 50% SAS and dialyzed against PBS. Protein concentrations were determined using the method of Lowry et al. (1951). The Mabs were filter-sterilized (0.45 μ), mixed 1:1 with glycerol, and stored at -20°C.

Biotinylation of selected Mabs using the method of Kendall (1983) was the final step needed for the Mab-based ELISA. This step facilitated the amplification of signal in the ELISA using the biotin-streptavidin system. After biotinylation the Mabs were filter-sterilized (0.45 μ), mixed 1:1 with glycerol and stored in a foil-wrapped tube at -20°C.

Capture or sandwich ELISAs were conducted using methods described by Turaga et al. (1987a) with the substitution of complementary mouse Mabs for rabbit antibodies. Different combinations of Mabs at equal protein concentrations were used in these assays, and multiple antibody analyses were also conducted. Mabs were ranked on the basis of their ability to produce high signals with minimal background. Analyses of antibody competition were conducted using the methods of Wiens and Kaattari (1989).

II. Development of Quantitative/Clinical ELISAs

The ELISA Based on Polyclonal Antisera. The Seattle laboratory uses the ELISA developed by Pascho et al. (1987). This assay utilizes commercially prepared reagents and buffers (Kirkegaard and Perry Laboratories, INC., Gaithersburg, MD) and a completely automated dispensing and washing system (Titertek and Titertek Autodrop, Flow Laboratories, McLean, VA). Optical densities (OD) were measured using a Titertek Multiskan Plus microplate reader (Flow Laboratories) with an interface cable to a Macintosh SE computer containing Delta Soft ELISA analysis software. In addition to comparison of different preparations of polyclonal antisera, development of this assay has also included evaluation of: different brands of ELISA plates for consistency and binding ability, antigen incubation time, the effect of heat-treated (15 min in a 100°C water bath) versus untreated tissue

samples on ELISA results, the diluent used in sample preparation and the effectiveness of sonication in sample preparation.

The ELISA Based on Monoclonal Antibodies. After the initial production and analyses of different Mabs, the Corvallis laboratory proceeded to develop an ELISA using Mabs for detection of antigen F in diseased fish. Several parameters of the ELISA were addressed in order to optimize the assay. These included antibody concentrations, incubation and wash buffers, and incubation times. The present report incorporates the information from the optimization analyses but does not include the results of tests leading to this optimization. Kidney and spleen samples were mixed 1: 1 with PBS and homogenized in a Stomacher-80 (Tekmar Co. Cincinnati OH) for 1 min. The homogenates were centrifuged and the supernatant used in the ELISA. Tissue fluids were loaded directly onto the plate. All manipulation of reagents except for addition of antigen was performed with an Autodrop Plate Loader (Flow Laboratories, McLean, VA). Replicates or triplicates of standard dilutions of soluble antigen (50 μ l each; 1-37.5 ng/ml) and replicates of dilutions of tissue samples were loaded onto the plates (50 μ l/well) using hand pipettors. All washes were performed using a Titertek Microplate Washer 120 (Flow Laboratories) with TTBS as the wash buffer. Horseradish peroxidase-conjugated streptavidin was produced commercially (Sigma Chemical Co. St. Louis, MO). Optical densities (**A₄₀₅**) 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 F in each tissue sample was calculated from the average optical densities using this linear equation. Dilutions were performed on high positive tissues to samples with optical densities within the standard curve. Standard curve data producing equations with correlation coefficients of less than 0.96 were not used for the calculations.

III. Western Blot Analysis

Western blot analysis of *R. salmoninarum* soluble proteins was performed to determine if this system can be used to identify false positives

in questionable ELISA samples. This was deemed essential as a result of previous evidence of *R. salmoninarum* antisera cross-reacting with other bacterial antigens (Dixon 1986; Yoshimizu et al. 1987). We therefore examined the sensitivity of blotting procedures using both polyclonal and monoclonal antibodies as probes. Dilutions of known concentrations of soluble protein produced from in vitro culture were electrophoresed and blotted using a Bio-Rad Mini Protean II electrophoresis unit (Bio-Rad Laboratories). After transfer, blots were blocked with 1% bovine serum albumin in TTBS (0.1% tween 20, 50 mM tris pH 7.2, 0.75% NaCl) for 1 h at room temperature followed by a single wash. Blots were then placed in specific polyclonal antisera or monoclonal antibody and incubated 1 h at room temperature. Unless indicated, all Mabs used in these experiments were produced from in vitro culture of hybridomas. After incubation in primary antibody the blots were washed 3X in TTBS. Peroxidase-conjugated goat anti-mouse antisera (Hyclone Laboratories, Logan UT) was added to the blots, incubated 1 h, and removed with 3 TTBS washes in TTBS. Blots were developed with Color Development Reagent (Bio-Rad Laboratories) and hydrogen peroxide and the development was stopped with extensive washing in tap water. Parallel blots were developed for total protein using colloidal gold protein stain (Aurodye Forte, Integrated Separations Systems, Hyde Park, MA).

Several procedures were used in attempts to enhance the sensitivity of the Western blot. One of these was the use of sodium acetate:ethanol to concentrate the amount of protein per volume loaded on the gel (Johnstone and Thorpe 1987). This method was ineffective as it increased the amount of non-BKD proteins in tested tissue samples and resulted in high background interference (not shown). A second method involved the use of gold-conjugated second antibody in the immunoblotting steps to enhance the sensitivity of the detection systems.

IV. Use of the ELISAs to Examine Naturally and Experimentally Infected Fish

Investigation of Carson National Fish Hatchery Adult Spring Chinook Salmon Using the ELISA Based on Polyclonal antisera.

Although many fluids and tissues may be used to monitor BKD infection levels in fish, it is still unknown which samples are most useful as a basis for gamete selection. Because this project involves assessment of infection levels at several life stages of the fish, it is important that ELISA protocols be developed for each tissue sample assayed. To compare the amounts of antigen present in different tissue samples and to assess the reliability of the polyclonal ELISA in detecting this antigen, samples of plasma, ovarian fluid, milt, kidney and spleen were collected from 350 adult Spring Chinook salmon during spawning at Carson National Fish Hatchery (Figure 2). Because these tissue samples varied in their composition, different methods of processing were employed for evaluation by ELISA. The following is a description of the methods used.

Segments of kidney (approximately 4 cm x 15 cm) were removed from the fish, held on ice, then stored at -20°C until processing. After thawing, tissue was thoroughly homogenized. Two samples were taken from the homogenate; both were diluted 1:3 (w/v) in PBS-Tween 20, one sample was heated 15 min and the second was vortexed. All samples were incubated overnight at 4°C, centrifuged at 10,000 x g (10 min at 4°C), and the supernatant analyzed by ELISA. A portion of each unprocessed tissue sample was also examined using fluorescent antibody techniques (FAT) so that the ELISA results could be compared to actual bacterial counts. Whole spleens were collected and processed by the same procedure employed for kidney tissue. Plasma was processed from whole blood which was collected from the caudal vein using heparinized syringes. The blood was centrifuged at low speed, and plasma collected and frozen (-70°C) until further processing. Duplicate plasma samples were diluted 1:3 (v/v) in PBS-Tween 20. Heat-treated and untreated samples were centrifuged and the supernatants analyzed by ELISA. Milt was collected directly from spawning males and refrigerated until processing. Duplicate samples were diluted 1:3 in PBS-Tween 20 and distilled H₂O, heat-treated, centrifuged and the supernatants analyzed by ELISA. Ovarian fluid was collected directly from spawning

females and refrigerated until processing. An aliquot of each sample was diluted 1:3 (v/v) in PBS-Tween 20, heat-treated, centrifuged and analyzed by ELISA. Another portion was frozen at -70°C for examination by filtration FAT (Elliott and Barila 1987).

Analysis with the Mab-Based ELISA of Samples Harvested from Adult and Juvenile Salmon. After development of the Mab-based ELISA, tissues from fish were examined for antigen load. We first examined tissues from experimentally infected coho and chinook salmon. These fish were infected by intraperitoneal injection of 0.1 ml of a 1.0 OD (A450) suspension of PBS-washed *R. salmoninarum* cells grown in KDM-2 + 10% bovine serum. Serum, kidney, spleen and liver samples were examined. Blood was collected from the caudal vein of fish killed by concussion. Samples were allowed to clot overnight at 4°C and sera were collected the next day. Kidney, liver and spleen samples were mixed 1:1 (w/v) with 4°C PBS and homogenized. Supernatants were collected after centrifugation (4°C, 1800 x g) and stored at -20°C prior to analysis with the ELISA.

Quantitative assays were performed on tissues from fish collected from 3 facilities in Washington and Oregon. Spring chinook populations returning to Carson National Fish Hatchery in Skamania County, WA and the Minto Collection Facility of the Marion Forks Hatchery in Linn County, OR were examined. Coho salmon returning to the Fall Creek Fish Hatchery in Lincoln County, OR were also examined. This sampling was important for further testing of the monoclonal-based ELISA system.

V. Selection and Modification of Hatcheries Suitable for the Segregation Experiments.

Of primary importance to this project was the selection of the two hatcheries for the segregation experiments. Each laboratory was to identify a hatchery with the following optimal criteria:

1. A pathogen-free water supply
2. A history of BKD among juvenile fish
3. At least 800 returning adult spring chinook
4. the ability to hold 400 erythromycin-injected and uninjected adults
5. 1 x 1 mating and individual egg incubation units
6. Space for installation of 80 individual rearing units for fry
7. Ability to provide 8 raceways for rearing until release
8. Freedom from other compromising diseases (e.g., viruses)

The minimum requirements were the following:

1. A history of BKD at the hatchery
2. At least 200 returning adult Spring Chinook
3. 1 x 1 mating and individual egg incubation units
4. Space for installation of 80 individual rearing units
5. Ability to provide 4 raceways for rearing until release

Agencies responsible for hatcheries on the Columbia River and its tributaries were contacted by each laboratory in order to identify facilities which satisfied as many of the optimal characteristics as possible. A selection was then made on the basis of these criteria.

RESULTS AND DISCUSSION

I. Standardization of ELISA Reagents

Production and Comparison of *R. salmoninarum* Antigens. The comparison of different culture media for production of antigenic proteins from *R. salmoninarum* strain D6 showed that the presence of serum in the medium was the major factor effecting the quality of the antigen produced. Analysis by Western blot of antigen from cultures grown in media containing serum, KDM-1 and KDM-2 S, demonstrated the predominance of the 57 kdal antigen F protein. In cultures lacking serum, breakdown products of this antigen resulted (Figure 3). Although growth in medium containing serum produced a large amount of intact antigen F protein, total protein determinations of these preparations reflected the serum from the medium and not proteins of *R. salmoninarum* origin. This problem eliminated KDM-2 S for the production of a standard soluble protein preparation for use in a quantitative ELISA.

Optical density (A_{405}) readings produced from the ELISA showed that total protein values of supernatants media did not correspond to the amount of antigen present. Supernatants from media without serum had higher antigen levels than would have been expected from protein values (Figure 4). For example, high ELISA values for supernatant from biphasic culture were not expected from the relatively low total protein levels; however, this medium contained little other protein. KDM-2 UF, while producing antigen without a large amount of media proteins, did not support much growth and was both labor and cost intensive.

Cell pellets produced from growth of both strain D6 and 33209 in the different media were weighed and examined qualitatively by Western blotting. Cell growth paralleled the production of antigenic protein (Table 1). Media containing serum produced the largest cell pellets, but serum components were hard to remove even with multiple washes. This problem was reflected in the electrophoretic profile of cell proteins (Figure 5). Electrophoresis of samples from cell pellets grown in media lacking serum had less complicated protein patterns, but there were several low molecular weight proteins present which were absent in cells from media with serum. It is likely that these proteins were antigen F breakdown products. Cell pellets

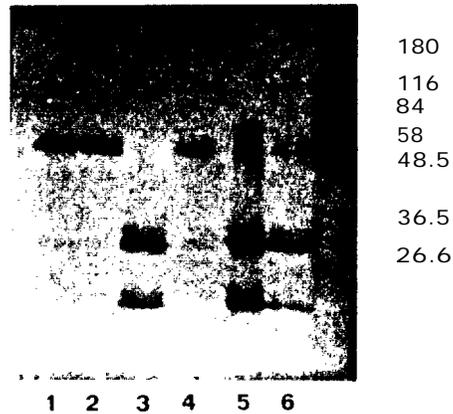


Figure 3. Western blot of SAS-precipitated supernatant antigens from 20 d cultures of *R. salmoninarum* strain D6 grown in (1) KDM-1, (2) KDM-2 S, (3) KDM-2, (4) KDM-2 UF, (5) KDM-2 C, and (6) biphasic medium. Protein concentrations were standardized to 0.1 ug/lane and the blot was probed with polyclonal anti-*R. salmoninarum*. Molecular weights of reference standards are indicated in kilodaltons.

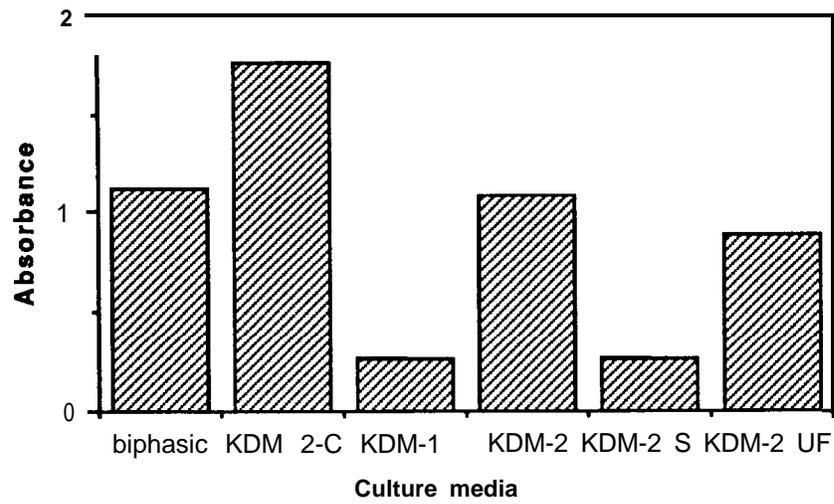


Figure 4. ELISA OD values of samples of SAS-precipitated proteins from *R. salmoninarum* strain D6 grown in six different media. All samples were standardized to 1 ug/ml total protein.

Table 1. Weights of cell pellets from *R. salmoninarum* strains D6 and 33209 grown in six different culture media.

Media ^a	Strain D6		Strain 33209	
	wet weight of <u>cell pellet</u>	incubation time	wet weight of <u>cell pellet</u>	incubation time
KDM-1	3.70 g	21 d	1.92 g	21 d
KDM-2 S	3.29 g	20 d	1.60 g	21 d
KDM-2	1.61 g	21 d	1.36 g	22 d
KDM-2 UF	0.18 g	22 d	1.08 g	26 d
KDM-2 C	1.51 g	21 d	1.43 g	22 d
BIPHASIC	2.07 g	22 d	0.64 g	17 d

a Volume of culture media was 500 ml except for biphasic medium which consisted of 200 ml peptone-saline overlaying charcoal agar.

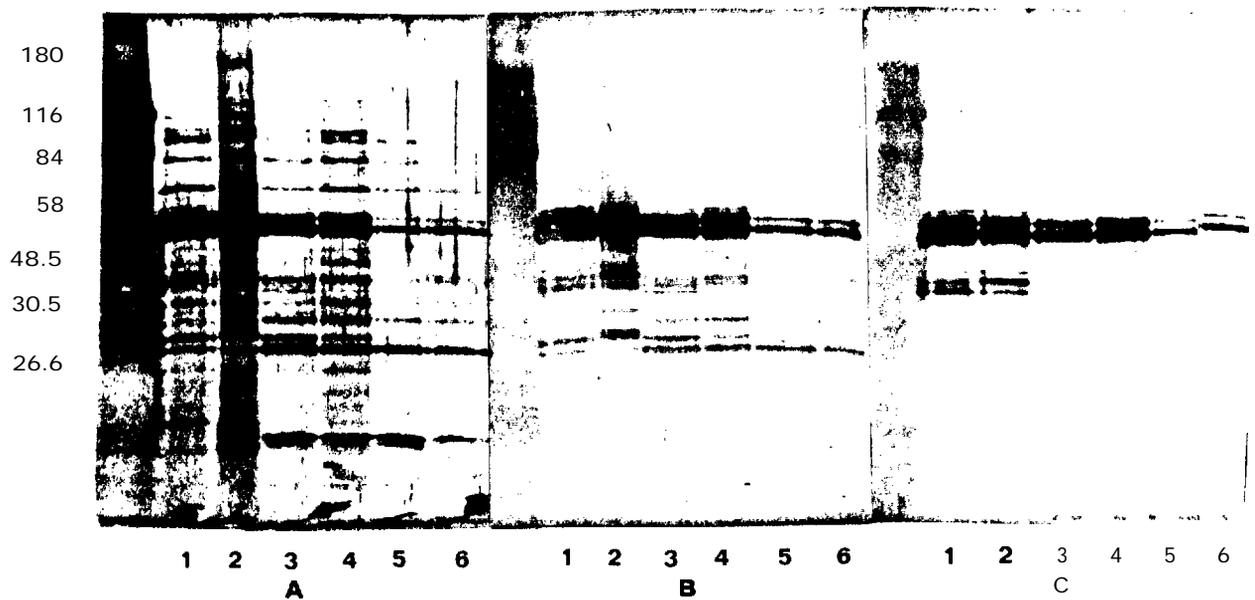


Figure 5. Western blots of *R. salmoninarum* strain 33209 cells grown in six different media; (1) KDM-1, (2) KDM-2 S, (3) KDM-2, (4) KDM-2 UF, (5) KDM-2 C, and (6) biphasic medium. Transfers were probed with (A) colloidal gold total protein stain, (B) polyclonal anti-*R. salmoninarum* and (C) Mab 4D3.

from KDM-2 and KDM-2 C were similar. Both media yielded smaller cell pellets than those from serum-containing media. Strain D6 grew best in the biphasic medium, producing a pellet of higher weight;volume ratio than any other medium. This result was not repeated for strain 33209, possibly because that culture was harvested earlier.

The quality and quantity of antigen prepared by water washing *R. salmoninarum* cells (Daly and Stevenson 1987) was also examined. High yields of antigen F with low concentrations of breakdown products were achieved using this method.

Results of the growth curve study show that although both KDM-2 and KDM-2 S supported growth of the bacteria, there were differences in the rate and amount of bacterial growth and in the protein composition of the antigen produced. Estimates of the number of colony forming units (CFU)/ml media showed that bacteria reached log phase growth between 4-6 d post-inoculation in KDM-2 S and between 6-8 d in KDM-2 (Figure 6). ELISA OD values for dilutions of supernatant showed that soluble antigen production paralleled CFU counts (Figure 7). Optical density values reflecting soluble protein concentrations for KDM-2 S supernatant increased sharply between 8-10 d post-inoculation then leveled off and decreased slightly by day 20, while OD values from KDM-2 increased between 12-14 d and peaked at 16 d before declining. Examination of samples of culture supernatant by SDS-PAGE and Western blot analysis showed that supernatants from KDM-2 S cultures showed a high ratio of medium components to antigen while supernatant from KDM-2 contained mostly *R. salmoninarum* antigenic proteins (Figure 8). As the age of the culture increased, the relative quantities of the 57 kdal antigen F protein produced in the media lacking serum decreased with respect to the concentration of breakdown products. However, the ratio remained constant in KDM-2 S.

In a similar experiment designed to investigate the production of antigens by *R. salmoninarum*, the protein profiles of culture supernatants from media lacking serum were examined at two different intervals after culture. *Renihacterium salmoninarum* strain 33209 was inoculated into KDM-2, KDM-C, and KDM-UF and harvested at 10 and 17 d. Supernatants were SAS-precipitated and analyzed by Western blotting. Results of this experiment illustrate that antigen F produced in media lacking serum were reduced to breakdown products in cultures harvested after stationary phase (Figure 9),

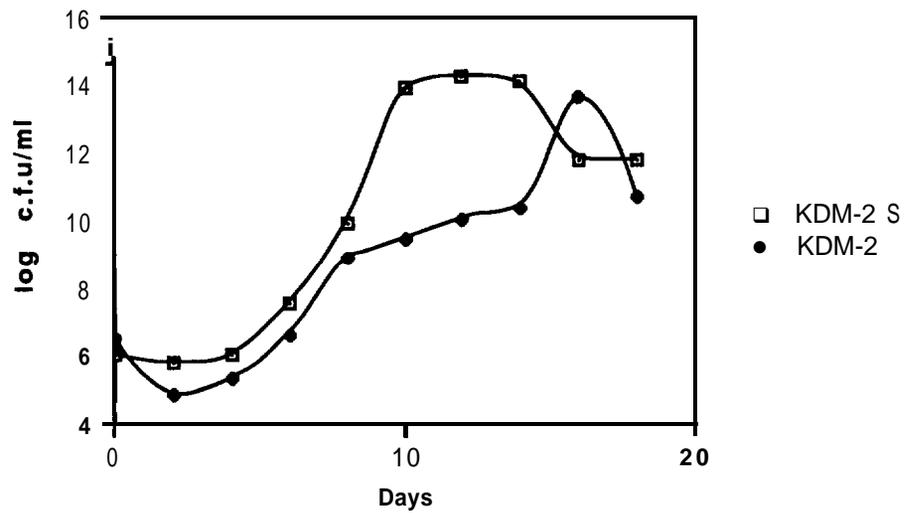


Figure 6. Bacterial growth estimates for *R. salmoninarum* strain D6 grown in either KDM-2 S or KDM-2 over a 20 d incubation period.

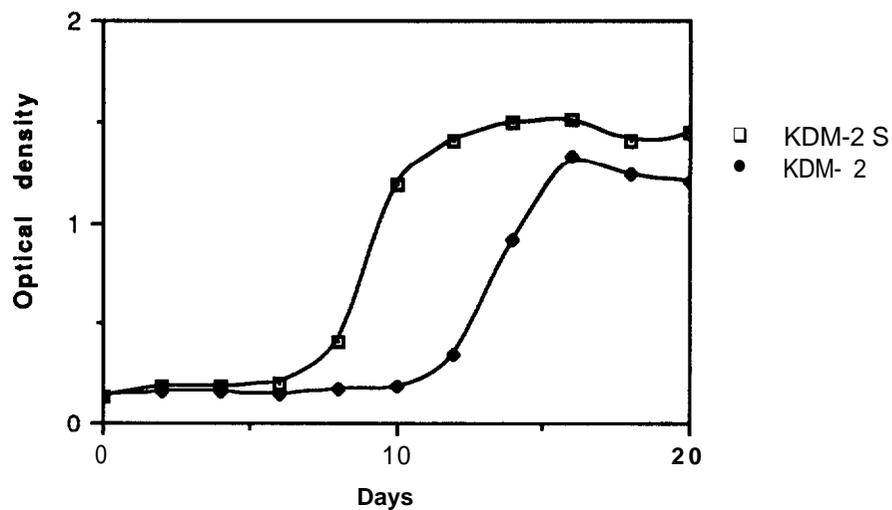


Figure 7. Optical density (A405) values from 1:100 dilutions of supernatants harvested from cultures of *R. salmoninarum* strain D6 grown in KDM-2 S and KDM-2. Values were determined with the polyclonal ELISA.

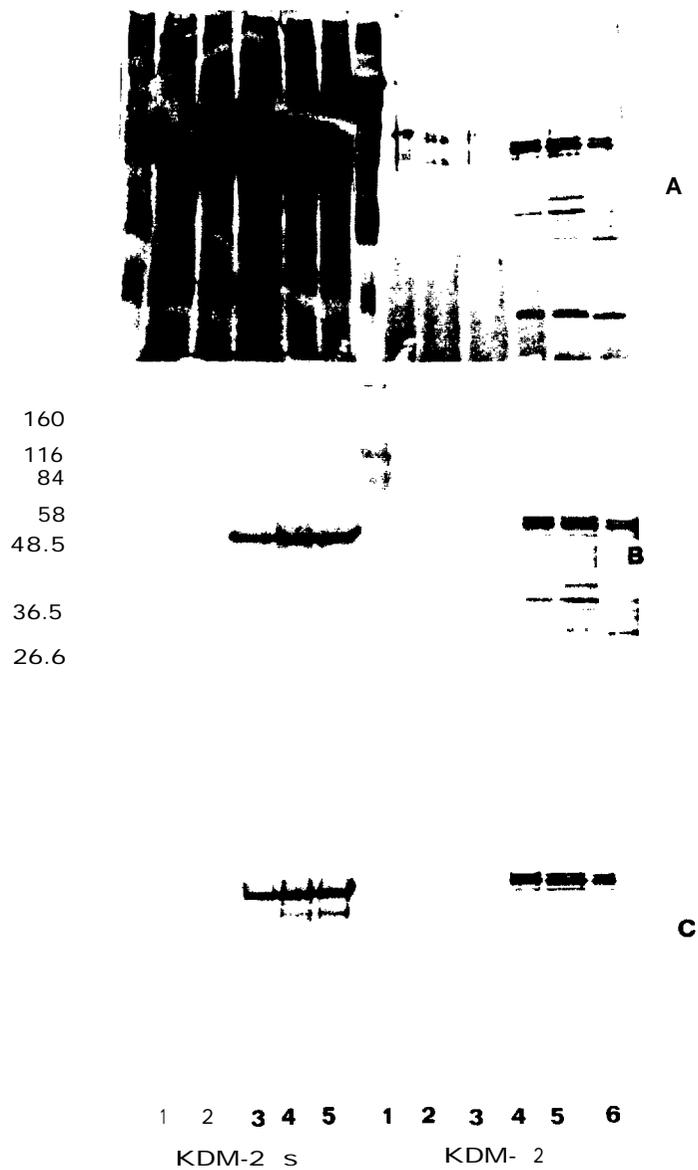


Figure 8. Western blots of supernatants from cultures of *R. salmoninarum* strain D6 grown in KDM-2 S and KDM-2 at five time intervals; (1) 2 d, (2) 6 d, (3) 10 d, (4) 14 d, (5) 18 d and (6) 20 d post-inoculation. (A) blot stained with colloidal gold total protein stain, (B) blot probed with polyclonal anti-*R. salmoninarum* and (C) blot probed with mab 4D3.

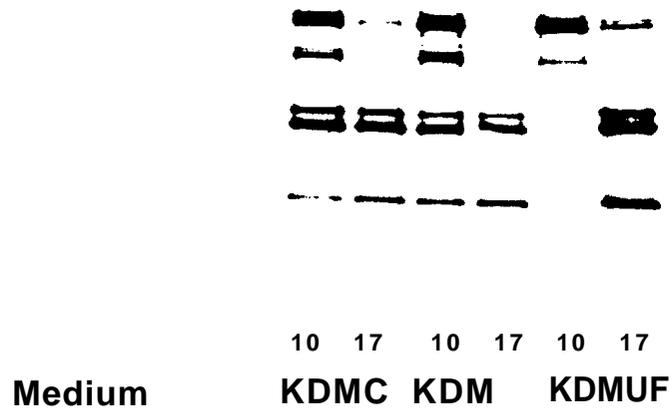


Figure 9. Western blot of SAS-precipitated supernatants of cultures of *R. salmoninarum* strain 33209 at 10 and 17 d post-inoculation in three different media; (A) KDM-2 C, (B) KDM-2 UF and (C) KDM-2. The blot was probed with Mab 4D3 and 3H1.

but that by harvesting in late log phase or early stationary phase it was possible to enrich for the intact protein.

Production and Comparison of Polyclonal Antisera. Eight lots of rabbit antisera and two of goat antisera produced against *R. salmoninarum* were acquired from selected federal, university and private laboratories. Western blot analysis of the antisera showed that both rabbit and goat antisera detected a large number of bacterial antigens. Most lots of antisera reacted similarly, regardless of the form of *R. salmoninarum* antigen (whole cell or soluble) or the route of injection. Differences in antigen recognition appear to result from differences in antibody concentration rather than differences in antibody specificity. In Western blots designed to detect the cross-reactivity of the antisera with other bacteria, only two of the rabbit polyclonal antibodies cross-reacted, one with *R. dentocariosa* and another with *A. salmonicida*.

These results indicate that polyclonal antisera can be effective in detection of BKD antigens and that the method of production of the antisera may not be as crucial to its use as the antibody concentration. However, all lots of antibody used should be tested for cross-reactivity against other bacterial antigens.

Production and Characterization of Mabs. Both laboratories had produced Mabs prior to the initiation of the project (Table 2). Additionally, Mabs were acquired from other laboratories and commercial sources. Certain Mabs of this initial collection are described in the literature (Wiens and Kaattari 1989, Newman et al. 1989, Sommer et al. 1989). Mabs described by Arakawa et al. (1987) were unavailable for analysis because attempts to reculture frozen hybridomas were unsuccessful. Additionally, 8 new Mabs were produced by the Corvallis laboratory this year.

Characterization of the Mabs by Western blot yielded three major groups (Figure 10) which recognize unique bands corresponding to different breakdown products of the antigen F protein. Each group reacted with a common spectrum of antigen F breakdown products. Additionally, group 1 Mabs recognized a unique 20 kdal protein, and group 3 a unique 25 kdal protein (Table 2). Group 2 Mabs did not recognize either of these proteins. Monoclonal antibodies of uncertain classification may define a fourth group because they appear to react only with the 57 kdal band and not with any breakdown products (not shown). Technical problems associated with Mabs

Table 2. Characteristics of tested **monoclonal** antibodies.

<u>Designation</u>	<u>heavy chain</u>	<u>light chain</u>	<u>Source</u>
<u>Group I</u>			
4D3	IgG1	k	Corvallis^a
4H8	IgG2b	k	Corvallis
4C11	IgG1	k	Corvallis
2A7	IgG2b	k	Corvallis
<u>Group II</u>			
2G5	IgG1	k	Corvallis^a
3H1	IgG1	k	Corvallis^b
<u>Group III</u>			
2G9	IgG2b	k	Corvallis
1A1	IgG1	k	Corvallis
4D1O	IgG1	k	Corvallis
2G9A	IgG1	k	Corvallis
A4B3	IgG3	l	Sommer^c
A9C3	IgM	NA^d	Sommer
RS04	IgG1	NA	Newman^e
<u>Uncertain classification</u>			
ID2	IgM	NA	Corvallis
710E	IgM	k	Seattle^f

a Described in Wiens and Kaattari 1989.

b Ole Bendik Dale of the National Veterinary Institute in Oslo, Norway, assisted in the production of this Mab.

c Described in Sommer et al. 1989.

d Not Available.

e Produced by Microtek Research and Development Ltd., Sydney, B. C., Canada. Described in Newmann et al. 1989.

f Produced by Ron Pascho, National Fisheries Research Center, Building 204, Naval Air Station, Seattle, WA. 98125.

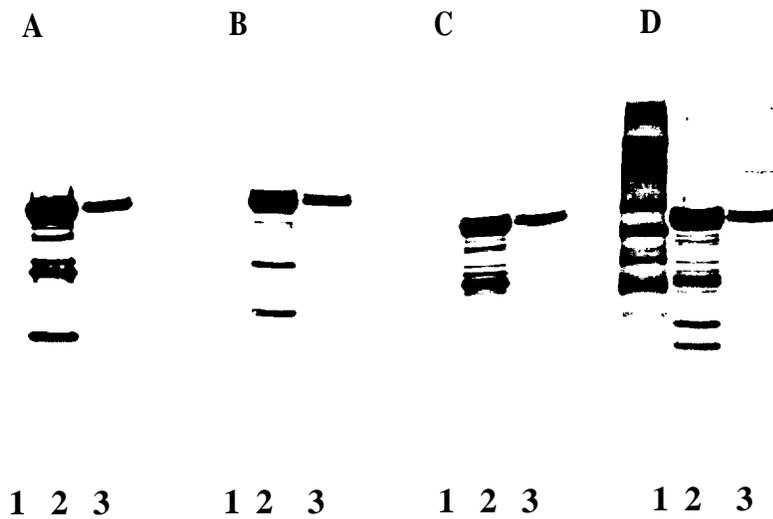


Figure 10. Western blots of *R. salmoninarum* antigens probed with: (A) Mab 4D3. (B), Mab 1A1. (C), Mab 3H1. Blot D is stained with colloidal gold total protein stain. In each blot, lane 1 is protein standards, lane 2 is soluble protein, and lane 3 is cell-associated antigen.

concentration and isotopes have caused their accurate classification to be undetermined.

Use of Different Mabs in ELISAs. Results of Western blots which showed recognition of different epitopes of the 57 kdal protein indicated that combinations of different Mabs could enhance the sensitivity of the ELISA. Combinations of antibodies and pools of Mabs were tested in the ELISA and compared on the basis of high optical density values and low background levels. Combinations within groups (i. e. 4D3 and 2A7) were eliminated because previous work has demonstrated that such Mabs recognize the same segment of the antigen F protein (Wiens and Kaattari 1989). Such combinations are not complementary- the binding of antigen by the first antibody in the ELISA precludes the binding of antigen by the biotinylated second antibody. Complementary combinations of Mabs involve antibodies from two or more of the different groups.

After comparison of several Mabs, we determined that coating with 4D3 and developing with biotinylated 3HI was a superior combination when either total optical density within a positive samples or background levels were compared (Figure 11). In the future, Mabs developed in the second half of the year will be examined in the ELISA to determine if the system can be improved.

II. Development of Quantitative/Clinical ELISAs

Development of the ELISA Based on Polyclonal Antisera.

Several components of the existing polyclonal ELISA system were examined to determine what improvements might be possible. A comparison study between different brands and lots of ELISA plates examined the amount of non-specific binding of tissue proteins, variability between replicate samples, and cost. The results show that there was a great deal of variation between brands of plates. Alternate methods of sample processing were also studied. In tests examining the effects of heat-treatment of samples, the polyclonal ELISA recognized both heat-treated and untreated purified antigen equally, but OD values of unheated tissue samples did not always correspond to those of heated samples or FAT results from the same tissue. The duration of antigen incubation on the ELISA plate was also found to

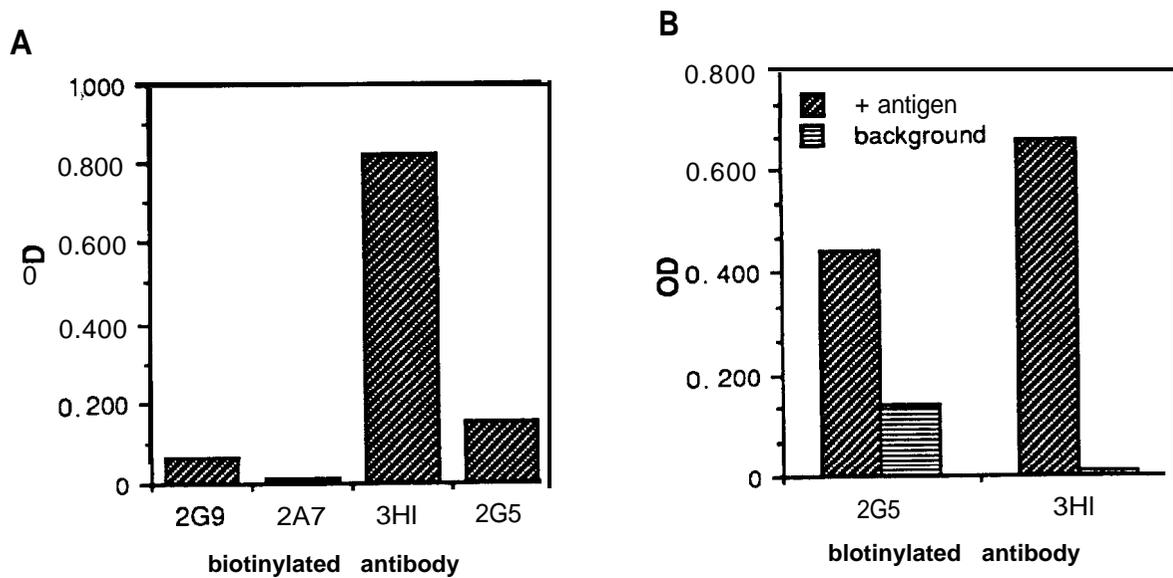


Figure 11. A. Average optical densities (A_{405}) of ELISA analyses using different biotinylated Mabs (1 ug/ml each) as second antibody. Mab 4D3 at 3.2 ug/ml was used as coating antibody and 50 ng/ml *R. salmoninarum* soluble protein was used as antigen. B. Comparison of the background optical densities when different biotinylated Mabs are used as second antibody in the ELISA. Assay conditions were identical to those described for panel A except that antigen was omitted for the “background” assays.

directly affect the results. As incubation time increased, ELISA OD values of all sample types increased linearly.

Milt samples collected at Carson hatchery were diluted in distilled water as well as PBS to compare release of antigen from sperm in the two diluents. However, use of distilled water caused release of the coating antibody from the plates, resulting in uniformly low OD values. The effects of sonication on samples processed for the ELISA was also examined because sonication was reported to be effective in releasing antigen from the tissues (Leek 1988). However, the effects of sonication of tissue prior to ELISA analysis was not as significant as those seen when tissues were disrupted by heating. Therefore, heating of samples will continue to be a standard practice in the polyclonal ELISA.

The polyclonal ELISA in use relies on dilutions of *R. salmoninarum* cells as the positive control. Although dilutions of cells are a good internal standard for assuring that all reagents are working, it has not been possible to use these standards quantitatively. The Seattle laboratory is currently examining alternative antigen preparations with the objective of developing a standard curve that is expressed in terms of protein concentration/ml of sample. This will allow better comparison of results from the polyclonal ELISA with results from the monoclonal ELISA. The procedure outlined in Table 3 is the polyclonal ELISA presently in use in the Seattle laboratory.

Development of the Mab-Based ELISA. The development of the standard ELISA involved the optimization of several different parameters. Concentrations of 1-5 $\mu\text{g/ml}$ were sufficient for either Mab. Incubation times, diluents and wash conditions were also investigated. The protocol presented in Table 4 summarizes the results of these analyses. It was necessary to establish antibody concentrations for each of the Mabs in the system (4D3 and biotinylated 3HI) which facilitated both high OD's and economical use of reagents.

Development of a consistent and reproducible standard curve for use in the assay was also very important. This allowed the comparison of a given optical density reading from a sample of tissue to samples analyzed on different occasions. The standard curve developed by the Corvallis laboratory covers a range of 1-37.5 ng/ml and can be fit by simple linear regression analysis (Figure 12). The sensitivity of this assay is such that many clinical samples require dilution prior to analysis.

Table 3. Steps in the ELISA based on polyclonal antisera.

Reagent a	Volume; concentration	Incubation temperature	Incubation duration	Wash b
Coating Ab c in PBS	200 µl/ 1 ug/ml	7°C, humid	overnight	5x
antigen	200 µl; unknown	room temp	2 hr humid	5x
Conjugate Ab d in diluent	200 µl; 0.05 µg/ml	room temp humid, dark	2 hr	5x
Substrate e	200 µl	35°C, humid	20 min dark	no
SDS Stop f	100 µl; 2.5%	plates read immediately after addition of stop solution		

a Reagents used in the Polyclonal ELISA are commercially produced by Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD.

b Wash Solution: 0.002M Imidazole buffered saline solution with 0.02% Tween 20 (KPL #506301).

c Coating Antibody: Affinity purified antibody to *R. salmoninarum* (GOAT) (KPL #049691) at a concentration of 1 ug/ml in 0.01 M MPB (KPL #508401).

d Conjugate Antibody: Peroxidase labelled affinity purified antibody to *R. salmoninarum* (GOAT) (KPL #049691) at a concentration of 0.05 ug/ml in 0.1% non-fat dry milk borate buffer (KPL #508200).

e Substrate: equal volumes of 2,2'-azino-di[3-ethyl]-benzthiazoline sulfonate at a concentration of **0.6 g/l in glycine buffer (KPL #506402) and 0.2% H₂O in citric acid buffer (KPL #506402B)**.

f SDS stop solution: 2.5% dodecyl sulfate in water (KPL #508502).

Table 4. Steps in the ELISA based on monoclonal antibodies.

<u>Reagent</u>	<u>volume; concentration</u>	<u>incubation temuerature</u>	<u>incubation duration</u>	<u>TTBS wash</u>
Mab 4D3 in PBS	50 μ l; 3 μ g/ml	17°C	Overnight	no
BSA in TTBS	200 μ l; 0.01 g/ml	room temp	1 h	5x
antigen	50 μ l; unknown	17°C	2 h	10x
Mab 3H1 in TTBS	50 μ l; 1 μ g/ml	room temp	1 h	10x
streptavidin peroxidase in TTBS	50 μ l; 0.25 μ g/ml	room temp	1 h	10x
substrate	50 μ l a	room temp in the dark	15 min	—

a Substrate recipe: 0.015 % H2O2; 0.2 mg/ml ABTS (2 2'-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid) in 10 mM citric acid buffer, pH 4.0.

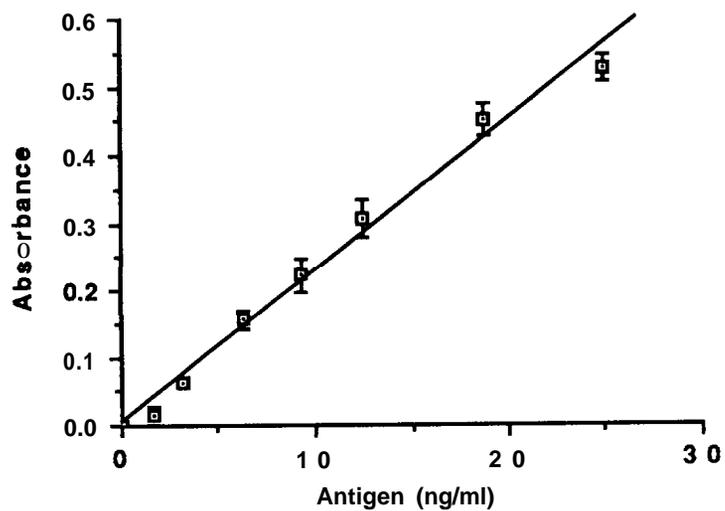


Figure 12. Average values of standard curves from 6 different plates using soluble protein concentrations from 1-25 ng/ml as antigen in the Mab-based quantitative ELISA.

III. Development of the Western Blot Assay

Antigen F was identified by Western blot in preparations of soluble protein diluted to approximately 1-10 ug/ml (Figure 13). This included analyses with both polyclonal antisera and monoclonal antibody probes, and two different signal development systems. The ELISA assay using monoclonal antibodies detects antigen F at concentrations as low as 1 to 3 ng/g of tissue. Similar results were obtained with the polyclonal system. This indicated that the ELISA was approximately 10^2 - to 10^3 -fold more sensitive than the Western blot. Therefore, identification of false positives in samples containing low concentrations of antigen cannot yet be accomplished by Western blotting. However, the Western blot is useful in detecting higher antigen concentrations and will be useful when the suspect tissue contains antigen concentrations above 1 $\mu\text{g/g}$.

Preliminary analysis of samples by Western blot using gold-enhanced immunodetection methods indicated that its sensitivity can be increased possibly 10 to 50 fold. In the future the use of this and other methods will be examined to provide a standard technique to verify results obtained using the ELISA.

The Corvallis laboratory used Western blotting to examine tissues from naturally infected fish. Kidney, sera and ovarian fluid from infected fish were examined. The major antigenic molecule was the 57 kdal protein reported by other authors (Turaga et al. 1987a; Wiens and Kaattari 1989). High molecular weight aggregates and breakdown products of the 57 kdal protein were also observed, and these varied from tissue to tissue in the same fish. Additionally, all bands visible in the infected tissues can be accounted for in soluble antigen prepared from in vitro culture (Figure 14).

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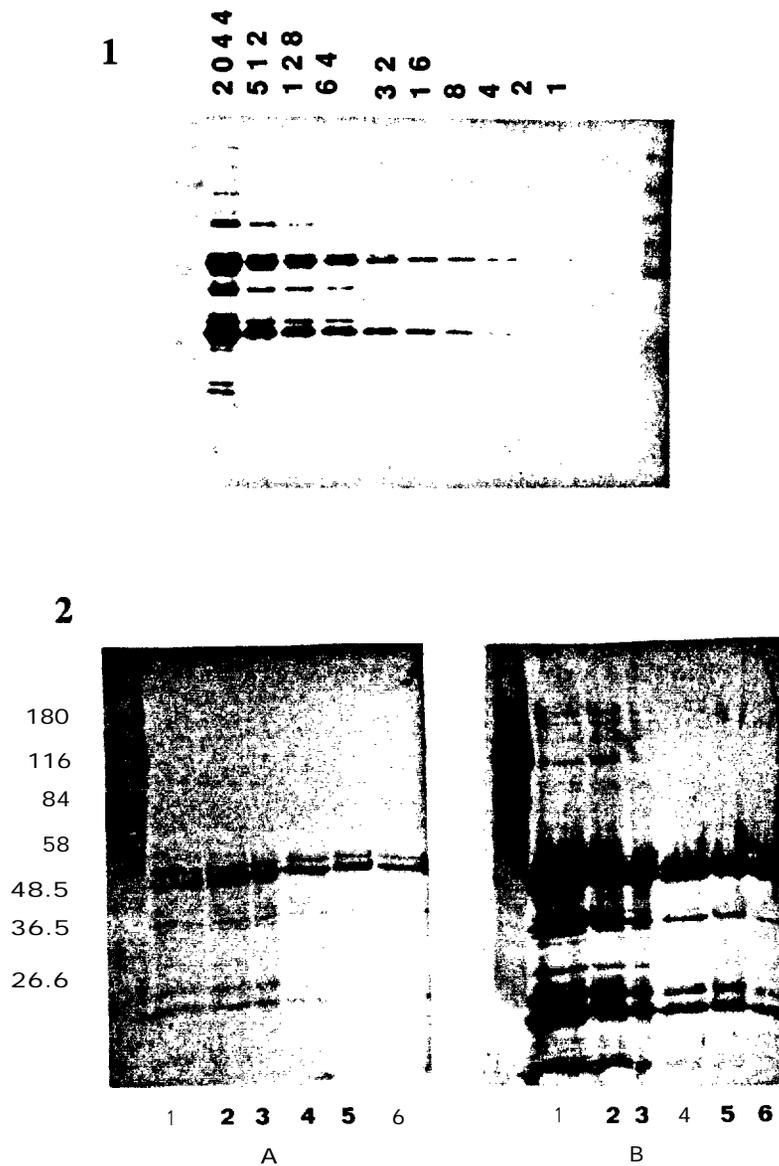


Figure 13. Examination of the sensitivity of Western blotting for the detection of *R. salmoninarum* soluble proteins. Soluble protein was produced in broth culture. 1. Blot probed with Mabs 4D3 and 3H 1 and developed with conventional peroxidase staining. Nanograms of protein indicated for each lane. 2. Blots probed with polyclonal antisera and developed with either conventional peroxidase staining (2A) or with anti-rabbit gold conjugates (2B). Total soluble protein in each lane for panel 2: 1; 2.5 μg ; 2; 1.25 μg ; 3; 0.625 μg ; 4; 250 ng; 5; 125 ng; 6; 25 ng.

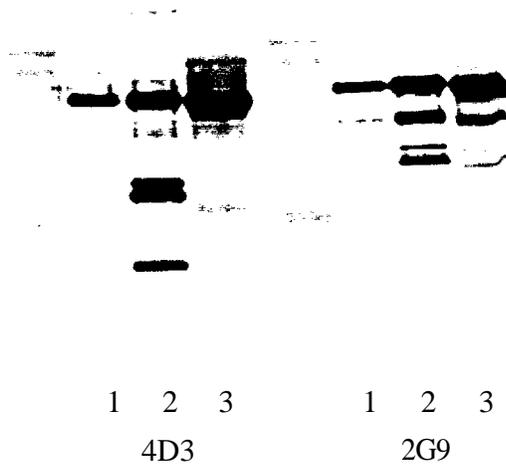


Figure 14. Western blots of: (1) Lysed *R. salmoninarum* strain 33209 cells. (2) Soluble protein from in vitro culture of strain 33209. (3) Kidney tissue from an infected adult spring chinook salmon. Blots were probed with the indicated Mab.



Figure 15. A. Western blot of *R. salmoninarum* soluble antigen from kidneys of chinook salmon returning to the Minto Collection Facility or from antigen harvested from in vitro culture of the bacteria. Antigen concentrations in the infected tissues (lanes 1-3) were determined with the ELISA assay. Concentrations for the soluble protein from in vitro culture (Lanes 5-7) were determined using the Lowry protein assay. Antigen amount/lane: 1) 306 ng (fish #159A. 2) 33 ng (fish #85). 3) 3 ng (fish #22), 4) kidney tissue with no detected antigen. 5) 300 ng. 6) 30 ng. 7) 3 ng. Lanes 8 and 9 are colloidal gold total protein stain of the highest concentrations in the Western blot. Lanes: 8) 300 ng antigen from broth culture. 9) antigen from fish #159A. St: molecular weight markers, values in kilodaltons.

examined to provide a standard technique to verify results obtained using the ELISA are comparable to the standard antigen preparation used for the calculations.

IV. ELISA-Based Examination of Naturally and Experimentally Infected Fish

Analysis of Samples from Carson National Fish Hatchery with the Polyclonal ELISA. Kidney, spleen, plasma, ovarian fluid and milt samples were collected from spawning adult spring chinook salmon at Carson National Fish Hatchery. ELISA OD values for kidney and spleen showed the greatest correlation and these tissues were chosen for determining antigen ranges and will be used for the segregation groups. Fish having OD levels greater than 0.2 for either tissue were considered heavily infected. This distinction, while arbitrary, is supported both statistically as being significantly higher than the values for negative fish, and by correlation with positive FAT results. Examination of the 241 female chinook salmon spawned revealed that 23 had ELISA OD levels greater than 0.2. ELISA OD values for sexual fluids did not always correspond to values of other tissues. Nine out of 109 males spawned were also heavily infected. In the male group, one milt sample correlated with high values for kidney and spleen; however, 16 fish were positive by milt alone. Among the females, antigen was detected in the ovarian fluid of 72 fish; however, the infection was confirmed in a different tissue type in only 8 of these cases.

Because high antigen concentrations in ovarian fluid may be an important indicator for vertical transmission of the bacteria, it is important to understand why the ELISA values of OF do not correlate with ELISA values from other tissue samples. Bacterial numbers determined by filtration FAT of OF samples positive are being compared with ELISA values. If bacteria are not detected, this may indicate that either some component of ovarian fluid interferes with the ELISA or that the ELISA is detecting soluble protein that is being released by a foci of infection not included in the tissue samples. In either case, eggs from these females may be excluded from the high infection level group. If, however, bacteria are detected, the eggs should be grouped with those from positive adults, The high number of females positive only for

OF may also indicate that there is contamination between these fluids during the spawning process. Thorough disinfection procedures will be included in the spawning procedure to ensure that cross-contamination at the hatchery will not be a problem.

The data collected from these samples was also used to determine what BKD antigen ranges can be expected at Carson Hatchery. The distribution curve for infected tissues was skewed heavily to the low OD range (Figure 16). A large number of adults with no BKD or with low infection levels were found while about 10% of the returning adults had antigen levels that were considered indicative of fish with moderate or heavy infection. Among this 10% positive group were fish with infection levels so high that the number of bacteria were uncountable by standard FATs and in which antigen levels were still observed in our highest dilution (> 1/100,000).

Analysis with the Mab-based ELISA of tissues harvested from returning adult salmon. Kidney, spleen, sera or plasma, and reproductive fluid were harvested from adult salmon returning to the Minto Collection Facility. Examination of these 5 tissues revealed that kidney and spleen yielded the highest concentrations of antigen F and level of detection while ELISAs of serum, plasma and ovarian fluid resulted in a lower rate of detection (Table 5). When kidney tissue was sampled from fish at other hatcheries on the Willamette River system (see map, Figure 2), similar percentages of the population demonstrated values above background. All fish that were positive by ovarian fluid or serum were also positive by kidney analysis. No antigen concentrations above background were detected using supernatant from seminal fluid (n=76) even when concentrations of antigen were detected in the kidney. Salmonid sperm has been shown to bind R. salmoninarum cells and this mechanism is likely mediated through the antigen F (Daly and Stevenson 1989). Antigen F protein present in semen may have high affinity for sperm as well and this may remove free antigen F from the environment. Boiling the milt samples as described by Pascho and Mulcahy (1987) is one possible mechanism for releasing this antigen. This is supported by the results of the Seattle laboratory presented in this report and will be examined for its application to the Mab-based ELISA.

Analysis with the Mab-based ELISA of spleens and kidneys collected at Carson National Fish Hatchery produced similar results. All fish with concentrations above background in the kidney were also above background

Table 5. Detection of *R. salmoninarum* antigen by monoclonal ELISA in tissues of adult Coho and Chinook salmon. Data is presented as a percentage with the total sampled in each group indicated in parentheses. Fish are listed as above background if the ELISA indicated a value of above 3 ng/g or 3 ng/ml in the tissue.

Sample							
<u>Location</u>	<u>Species/date</u>	<u>kidney</u>	<u>spleen</u>	<u>blood fluid</u> ^a		<u>milt</u>	
Minto	Chin./ 9- 19-89	10.3 (146)	N. S. b	s	7 (100)	6 (100)	0 (46)
	Chin./ 9-25-89	7.5 (120)	N. S.	P	4.2 (120)	3.3 (60)	0 (30)
	Chin. 9-29-89	11.8 (68)	7.4 (68)	P	5.8 (68)	5.9 (34)	N. S.
Carson	Chin./ 1 1/89	11.4 (147)	9.0 (223)	N. S.		N. S.	N. S.
Fall Ck	Coho/1 1-30-89	15.7 (84)	N. S.	S	6.0 (84)	N. S.	N. S.
				P	8.3 (84)		

a Either plasma (P) or serum (S) were sampled as indicated.

b N. S.; not sampled

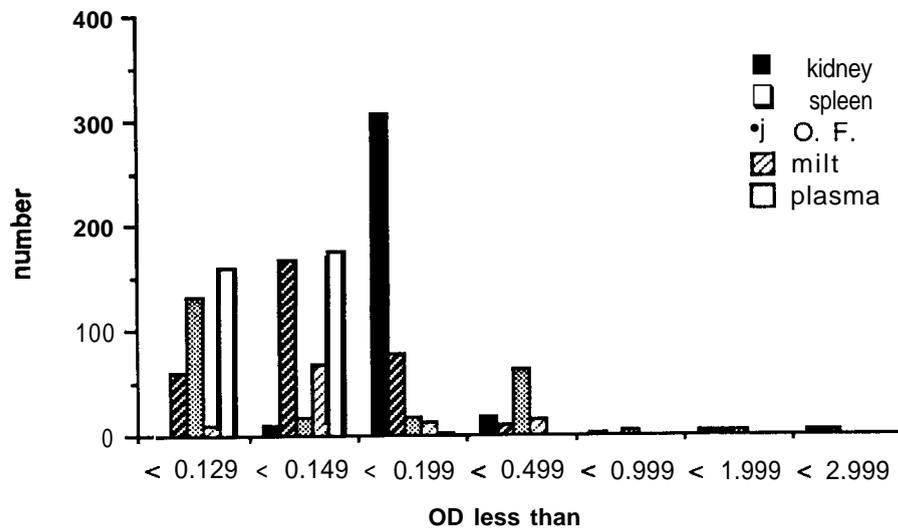


Figure 16. Optical density values derived from the polyclonal ELISA for selected tissues harvested from adult Spring Chinook salmon at the Carson National Fish Hatchery. One kidney sample not indicated on the graph was above 3.0 in the ELISA.

for the spleen. Exceptions were found only with concentrations of antigen below 3 ng/g

After identifying fish with OD values above background we determined the concentration of the antigen in the tissues. The antigen concentrations ranged from below background to a high in the kidney of over 200 $\mu\text{g/g}$ (Figure 17) and each of the three hatcheries examined had fish with antigen concentrations in the kidney above 50 $\mu\text{g/g}$. Preliminary results indicated that these concentrations could be found whether fish were treated or untreated with erythromycin (not shown). In the second year of the project, the segregation experiments will allow for the detailed analysis of the effect of erythromycin therapy on the accuracy of the ELISA.

Kidney samples of 180 fish from the Minto Collection Facility were also examined by FAT. All FAT positive samples with the exception of a single I+ sample was above background in the ELISA. A general correlation was observed between the antigen concentration by ELISA and the degree of infection as determined by FAT (Table 6).

Tissue Comparisons in Experimental Infections. Spleen, kidney and liver were harvested from moribund coho salmon which had been injected with *R. salmoninarum* cells. Antigen F was detected in each tissue (Figure 18). Controls indicated that background problems were evident in the liver samples. Analysis with the ELISAs of experimentally infected juvenile salmon will be a major focus of the second year of this project.

V. Selection of Hatcheries

Each laboratory has made the selection of the hatchery to be used in the segregation experiments. The Seattle laboratory will use the Carson National Fish Hatchery in Skamania County, WA. The Corvallis laboratory will use the Marion Forks Fish Hatchery in Marion County, Oregon (see map, Figure 2). Each facility meets the minimum requirements and satisfies most of the optimal criteria.

Carson National Fish Hatchery meets the minimum and approaches the optimal criteria established for the segregation experiment. Although there are no physical or chemical methods employed to provide water free of

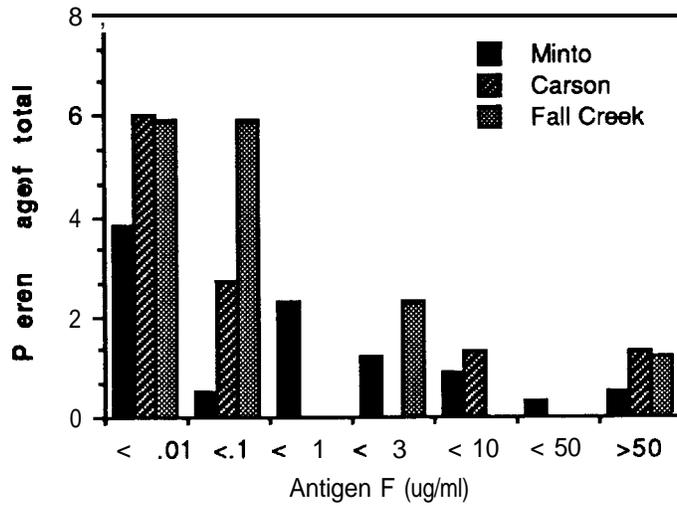


Figure 17. Range of soluble antigen concentrations in kidneys of adult Spring Chinook salmon returning to the selected hatcheries. Values were determined with the quantitative Mab-based ELISA.

Table 6. Relationship between antigen F concentrations in the kidney of infected adult salmonids collected at the Minto facility and their respective fluorescent antibody test (FAT) values.

FISH NO	Antigen F concentration	FAT
26	12.8 $\mu\text{g/g}$	3+/4+
27	12.3 $\mu\text{g/g}$	3+/4+
85	11.7 $\mu\text{g/g}$	4+
83	3.55 $\mu\text{g/g}$	4+
16	1.75 $\mu\text{g/g}$	4+
22	1.18 $\mu\text{g/g}$	1+
91	1.07 $\mu\text{g/g}$	4 t
142	192 $\mu\text{g/g}$	2+
134	175 $\mu\text{g/g}$	2+
98	81.1 ng/g	1+
3	6.64 ng/g	1+
77	6.24 ng/g	1+
20	5.35 ng/g	1+
119	3.9 ng/g	1+

Fifty nine samples negative by FAT and ELISA.

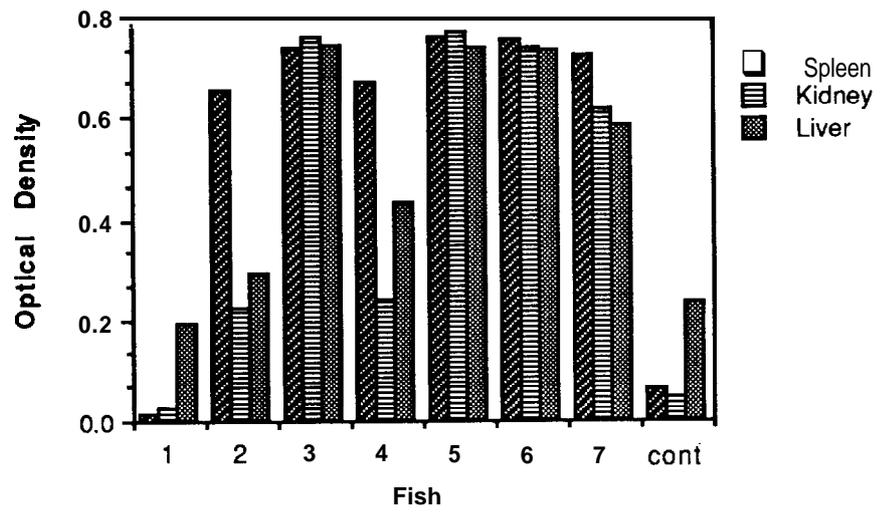


Figure 18. Optical density values from the Mab based ELISA for selected tissues harvested from 7 experimentally infected juvenile coho salmon and an uninfected control fish.

pathogens, spring water is obtained for use in the hatchery. Pathogenic microorganisms have never been detected in this supply. Historically, bacterial kidney disease has caused chronic, low level losses among juveniles, but the disease has not been a major problem. High percentages of returning adult fish have tested positive for infectious hematopoietic necrosis virus (sometimes over 90%), but losses in the fry have been sporadic and limited.

Delayed fertilization of gametes will not be possible at Carson because of hatchery production strategies. However, the large number of independent matings produced at each spawn will allow the identification of sufficient high and low crosses required for the four groups.

Marion Forks Hatchery is an equally suitable location for the segregation experiments outlined in this report. The water supply and clinical history of the facility are suitable and the numbers of fish returning to the Minto Collection Facility will be sufficient for the crosses. Some construction will be required to accommodate the progeny from the crosses. Independent egg rearing facilities and six foot circular tanks are being installed at the hatchery to accommodate the 1 x 1 matings and early rearing of the progeny.

A major emphasis of the first six months of the second year will be the preparation of the hatchery facilities for the segregation experiments. Time lines for each project have been established and are given in appendix one and two of this report. Modification of the hatchery facilities to accommodate the experiments have been discussed with appropriate hatchery and management personnel and will be completed at each hatchery prior to the spawning period.

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APPENDICES

Appendix one. Tentative Carson Hatchery Schedule for Year 2.

1. June 15-July 30, 1990. Hold 400 injected and 400 uninjected adults.

Fish will be tagged at time of injection (mid-June and mid-July) with color-coded and numbered Floy t-bar tags. Up to 1,000 injected fish and 500 uninjected fish will be tagged during each of the two injection periods. Fish arriving at the hatchery following the second injection will not be marked or injected. Samples representing fish from early, middle, and late arrivals (both injected and uninjected) to the hatchery will be necessary in order to avoid biasing the experimental results.

2. August 1990. 1 x 1 spawning.

Spawning begins the first week of August and continues four weeks. Individual fish will be numbered in order to identify samples collected during spawning. Spawning will probably be done on two successive days in order to obtain all of the necessary samples. If an insufficient number of males are available to accomplish 1 x 1 spawnings, milt from one male may be used to fertilize the eggs from two or more females. When using one milt take for multiple females, having the male's BKD level influence the female's level may cause the loss of some usable samples due to repetition.

To reduce contamination between fish, the person taking the eggs will wear a disposable glove on the hand holding the knife. The knife will be disinfected thoroughly between fish and a fresh glove worn for each fish. The eggs are spawned into individual containers (of which we will supply additional containers, providing single use per spawning to reduce the chance for contamination between egg groups), fertilized, hardened in iodophor, transferred to numbered containers, and placed in the incubation building. Hatchery personnel will track individual mating pairs from the spawning area until the eggs are replaced in the indoor facility. The hatchery manager has agreed to do a dry-run of the spawning procedure prior to August to work out unanticipated problems.

Our data should be processed prior to the second spawning and between each of the subsequent spawning periods so we can obtain adequate samples for the experiment (i.e., we need to know what the composition of our egg take should be at the time the fish are spawned).

3. Oct. 1990-Jan. 1991. Hold eggs as individual groups.

For hatchery purposes, eggs are held in containers until eye-up in October, when they are picked and combined into the egg trays and held in the troughs until swim-up in late December/early January, when they are moved into indoor tanks. The egg holding setup at the hatchery provides an individual water supply for each of the egg containers.

Our experiment will require 88 individual matings to fill eight raceways at densities of approximately 40,000 fish (average fecundity is 4,300, and raceway density should be about 40,000 fish, therefore, using 11 fish and assuming 10-15% loss should yield densities of 40,500-42,500). At the eye-up stage (October) we will have the 88 individual tanks in place to retain approximately 200 fish from each of our individual matings. Eggs will be held in breeding cages in the small tanks (need 5.6-7.6 square inches per 100 eggs; 6-7 mm diameter). The combined eggs for each raceway can be handled similarly to the hatchery procedure (i.e., picked-combined-trayed). The eggs from 11 females (1 raceway) will be divided into 2 stacks and the 2 stacks will be placed in 1 trough so each replicate group is maintained on a separate water supply. Fry will be sampled for ELISA both from the individual tanks and from the pooled groups when the families are ponded in the inside tanks, and again in 2 months.

4. May 1991. Ponds.

Our fish will be ponded in the outside raceways in early May (size approximately 180 fish/pound). The water supply for the eight ponds needs to come from the same source (can't have some raceways on spring water and some on river water). Feeding should follow hatchery practices. Hatchery does not normally feed antibiotics.

Fish are tagged at ponding with coded wire tags. All of our fish will need to be tagged (need eight individual codes one for each of the experimental groups). Ordering CWTs and coordinating tagging efforts needs to be done by May 1991. We will require 8 different coded wire tags and all fish to be released, approximately 320,000, must be tagged each year.

Juveniles will be sampled both from the outdoor ponds and the individual tanks every 3 months (or as long as the individual groups last).

5. April or May 1992. Release.

Fish will be released as yearlings.

Details of the segregation have been discussed with the acting hatchery managers and personnel of the hatchery, the US Fish and Wildlife Service biologist and the project officer of the tagging study that is also being conducted at the hatchery. Potential conflicts with that study have been solved by our agreeing to tag 100% of the experimental groups. Daniel Diggs, Associate Manager, Columbia River Basin, was contacted and he confirmed that the US Fish and Wildlife Service would cooperate with our objectives if our fish were uniquely tagged. Bill Shake, Region I, has been contacted and has expressed an interest in funding that portion of the study at Carson. Curtis Burley, Vancouver Fisheries Assistance Office, has been contacted about coordination of tagging efforts.

Appendix two. Flow chart and time course of the segregation experiments at Marion Forks Fish Hatchery.

Period beginning

Sept. 1, 1990 Water is diverted from the North Santiam River into the Minto Collection Facility and fish begin to enter the trap. Injection of fish with erythromycin begins and the injected fish (E group) are kept in a pen separate from other fish entering the facility.

Sept. 15, 1990 Regular spawning of fish uninjected with erythromycin (N group) begins at the hatchery. Overnight delayed fertilization will be conducted to allow identification of fish with the highest and the lowest antigen concentrations by ELISA. After the ELISA assays, information will be delivered to the hatchery and crosses will be made. After fertilization and water hardening, eggs will be placed in individual egg incubators for development. At each successive spawn the highest positives will be identified, and eggs from later spawns will be substituted for marginal eggs from earlier spawns. This will guarantee that enough eggs are harvested and that eggs will be collected from both the beginning and the end of the run.

A total of 335 fish returning to the Minto Collection Facility were examined this year of which 33 were positive. We anticipate high **returns to** Minto next year (Max Smith, ODFW, personal communication) and assuming a similar infection rate, we anticipate adequate fish supplies to conduct the crosses.

Sept. 20, 1990 Eggs will begin to be harvested from the E group fish per year. The procedure will be the same as above.

Nov. 21, 1990 Assay eyed eggs by ELISA

Jan. 15, 1991 Assay swim-up fry by **ELISA**

Feb 15, 1991 **Pool swim up fry into Canadian troughs inside the hatchery building or 6 ft circular tanks placed outside in hatchery ponds.** Triplicate sample groups within each major group will be kept independent throughout the rest of the culture of the fish.

- March 15, 1991 Move fish to 24 ft circular ponds. Assay 200 fish/pond every 3 months.
- July 15, 1991 Split fish to accommodate growth.
- Sept., 1991 Tag fish. Marion Forks Hatchery has experienced 9-15% losses in their spring chinook stocks in the past. If approximately 12% of our eggs die prior to tagging, we will need to tag 3 16,000 fish.
- March 1992 Release smolts and monitor returns.

The group in Corvallis has discussed the segregations in detail with Harry Lorz, Terry Jones, Rich Holt and Tony Amandi of the Oregon Department of Fish and Wildlife. Max Smith, Dennis Isaacs and Mary Buckmann (All of the ODFW) have also been contacted regarding specific aspects of the study.