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Abstract approved Dr. J. L. Fryer

The purpose of this project was to study the possiblity of controlling furunculosis in salmonid fish using immunological procedures.

Antiserum with an agglutinating antibody titer of 1:20,480 against Aeromonas salmonicida was produced in a horse. This hyperimmune serum was used to passively immunize coho salmon, Oncorhynchus kitsutch, (jacks). However, this work was discontinued, because the experimental animals were infected with a protozoan parasite (Ceratomyxa shasta).

Since a method of challenging the passive protection given adult coho jacks (Oncorhynchus kitsutch) was needed, a lethal dose of Aeromonas salmonicida cells was established. It was found that a 0.2 ml injection (intramuscular) of Aeromonas salmonicida suspension giving 20 percent transmittance at 475 mμ would kill 60 percent

of the test fish in 11 days.

Four year old adult chinook were actively immunized with two vaccine preparations, each injected by two routes. It was found that vaccine in adjuvant preparation injected by the intramuscular route was most effective. All methods of immunization induced the formation of agglutinating antibody. However, the vaccine in adjuvant injected intramuscularly produced the highest titer (1:1,280). An attempt was made to determine the optimal dose of vaccine in adjuvant when injected intramuscularly. No definite conclusion could be made, because most of the test fish died early in the experiment.

Active immunization of juvenile coho salmon was attempted by orally administering two different vaccine preparations. The first vaccine employed was formalin-killed Aeromonas salmonicida cells. Practical protection of the test fish against a natural epizootic of furunculosis was not accomplished by feeding the animals this vaccine in the normal diet for 37 days. The total number of cells given 1,000 fish was 7.1×10^{14}. The second vaccine was an alum-precipitated cell fraction of Aeromonas salmonicida. This preparation was also mixed with the normal diet (Oregon Moist Pellets) and administered at two levels, approximately 50 mg or 25 mg of vaccine per fish and at various time intervals before the epizootic. This alum precipitate was very effective for protecting the fish against a natural epizootic of furunculosis. Losses in two groups of fish which

were not immunized were 27.2 and 37.0 percent, while the highest loss in an immunized group was 0.7 percent. The two levels of vaccine administered (25 mg or 50 mg) did not affect the degree of protection afforded the fish. The data indicated that protection could be given if vaccination was accomplished at least 27 days prior to the onset of the epizootic.

Control of Furunculosis in Pacific Salmon by Immunization

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INTRODUCTION

Juvenile salmonid fish reared in hatcheries are necessarily subjected to crowded conditions creating an ideal environment for bacterial infections which may reach epizootic proportions. The same is true where dams block migration of the returning adult salmon. These animals are usually received and held in adult holding ponds until the advent of spawning operations.

One of the most important bacterial diseases of fish encountered in the production hatchery is furunculosis. The disease was first observed in hatchery fish by Emmerich and Weibel in 1894. An extensive review of the literature concerning this disease and the causative agent, Aeromonas salmonicida, has been prepared by McCraw (1952). The importance of furunculosis as a disease of adult Pacific salmon has been indicated by Fryer and Conrad (1965).

While the use of certain drugs for control of furunculosis is effective, high costs of treatment, drug-resistant strains of the bacterium and federal regulations concerning their use make it important to develop new and improved prophylactic measures for furunculosis. Immunization is one of the most effective prophylactic measures presently employed for control of infectious disease in

both human and veterinary medicine.

This research was initiated in an attempt to adapt existing techniques for immunization of fish and determine the feasibility of using induced immunity to control furunculosis in adult and juvenile Pacific salmon.

Passive immunization of juvenile salmon is not practical because of the time and expense involved in injecting large numbers of fish. However, it could be useful where a small number of adults are subject to disease. For this phase of the study, coho jacks[1] (Oncorhynchus kitsutch) were passively immunized with A. salmonicida antiserum produced in a horse and subsequently artificially challenged with the organisms.

Active immunization was studied in both adult chinook males (Oncorhynchus tshawytscha) and coho jacks employing an injected vaccine (antigen) prepared from killed cells of the bacterium. Antibody development in test fish was followed by measuring the appearance of agglutinating antibody in the serum.

Oral administration of the antigen is the only practical procedure for actively immunizing large numbers of juvenile fish. Juvenile coho salmon were used for oral immunization experiments

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[1] Sexually precocious male salmon which have matured before their age class. The length of these fish generally ranges between 18 and 20 inches.

performed at the Fish Commission of Oregon Siletz River Salmon Hatchery. This location was selected because the fish experience a natural, predictable epizootic of furunculosis which occurs about the first of June each year.

This study also includes some preliminary work designed to modify existing fluorescent antibody techniques for use in identification of fish pathogens. Aeromonas salmonicida, Chondrococcus columnaris, and the causative organism of bacterial kidney disease were employed in this portion of the investigation.

LITERATURE REVIEW

Since the original description of furunculosis in fish, the disease has received considerable study. However, only a small portion of this attention has been focused on the immunological aspects of the disease.

Drug Resistant Strains of *Aeromonas salmonicida*

The effective control of furunculosis by use of sulfonamides was first reported by Gutsell (1946). However, in little more than 10 years, strains of *A. salmonicida* highly resistant to sulfonamides were reported by Snieszko and Bullock (1957a). They proposed the following treatment schedule: sulfamerazine, sulfamethazine or a mixture of sulfamerazine and sulfagaunidine followed by a three-day treatment with chloramphenicol or oxytetracycline. In the same year that drug combination therapy was proposed, an *A. salmonicida* strain resistant to oxytetracycline was reported (Snieszko and Bullock, 1957b). Some immunological research was performed prior to the emergence of drug-resistant strains of *A. salmonicida* (Duff, 1942). This problem has, however, greatly stimulated interest in this area.

Passive Immunity to Aeromonas salmonicida

The possibility that immune serum of one fish could protect another against A. salmonicida infection was investigated by Spence, Fryer and Pilcher (1965). They injected a group of juvenile coho salmon with the immune serum (titer 1:320) from rainbow trout (Salmo gairdnerii) and another group with normal rainbow trout serum. Both groups were artificially infected with A. salmonicida. As a result of the induced infection, 72% of the fish which received immune serum and 90% of the fish which received normal serum died. This is the only reported investigation concerning passive immunity in fish.

Active Immunity to Aeromonas salmonicida Induced by an Injected Antigen

Gee and Smith (1941) injected three painted turtles (Chrysemys elegans) and one snapping turtle (Chelydra tricarinata) twice weekly with heat-killed A. salmonicida cells. The animals were held at room temperature ($23^{o}C$-$28^{o}C$). The authors state that no agglutinins were present in the blood at the beginning of the experiment. Each animal receiving the vaccine did develop agglutinins within six months, and a maximum titer of 1:2,560 was observed. They also reported that 27 adult carp (Cyprinus carpio) were given

semiweekly intraperitoneal injections of 1 ml of A. salmonicida vaccine ($5x10^9$ cells/ml) for seven weeks. The fish were held in water at 20°C-23°C, after which they were placed in flowing water at 10°C. Twenty-six of the test fish developed agglutinins. Twenty fish showed an increasing antibody titer as the injections continued, and these titers continued to increase after the fish were placed in water at 10°C. No control (nonvaccinated) carp showed agglutinins for A. salmonicida.

Smith (1940) placed five adult carp in each of three tanks containing flowing water at 10°C. He injected five fish weekly, five semiweekly and five biweekly. Each animal received the same amount of heat-killed A. salmonicida cells, 2 ml within two weeks ($5x10^9$ cells/ml). Most of the fish died within 12 weeks because of confinement. Of the eight surviving until the 10th week, agglutinating antibody titers ranged from 1:20 to 1:640. No control carp (non-injected) were shown to have agglutinating antibody titers. Rainbow and brown trout (Salmo trutta) were held at 10°C and given weekly injections of the same vaccine. Few of these fish survived longer than five weeks. Post-mortem blood samples showed brown trout to have titers as high as 1:640 while one rainbow trout had a titer of 1:1,280. Three control trout from a total of 74 were shown to have a titer of 1:80, which was probably due to previous exposure to the

disease./

Spence, et al. (1965) were able to induce agglutinating antibody titers as high as 1:640 in rainbow trout. The vaccine used was prepared from formalin-killed cells of A. salmonicida emulsified with Freund's (complete) adjuvant. The trout were injected with 2 ml of the vaccine followed by 1 ml injections one and six weeks later. All injections were administered intra-abdominally.

Krantz, Reddecliff and Heist (1964a) used formalin-killed cells of A. salmonicida to immunize brook (Salvelinus fontinalis) and brown trout. The trout were given a single intraperitoneal injection of either killed cells or killed cells in a mineral oil adjuvant. The results indicated that fish which received vaccine in adjuvant produced a higher (1:5,120) agglutinin titer than fish receiving killed cells alone (1:160). The fish which received vaccine in adjuvant retained a high titer (1:1,280) for a 10 month period, whereas the titer of fish receiving cells alone dropped to 1:10 after six months. Both groups of fish were challenged by injection of viable A. salmonicida cells. The mortality for those fish receiving cells alone was 23% to 38%; in the group which received vaccine in adjuvant, there were no mortalities.

Oral Immunization Against Aeromonas salmonicida

Duff (1942) showed that cutthroat trout (Salmo clarki) could be protected from furunculosis by feeding them chloroform-killed cells of A. salmonicida incorporated in the regular ration. He reported that providing the trout with 40 daily feedings of the killed cells mixed with the food failed to stimulate immunity; however, 64 or more feedings did. When challenged with A. salmonicida, 75% of the nonimmunized and 24% of the immunized fish died.

Snieszko and Friddle (1949) attempted to protect brook trout by feeding them heat-killed A. salmonicida cells mixed with the food (0.5 ml of packed cells/kg of food). When challenged with the virulent bacterium by two routes, oral or injected, none of the fish survived.

Krantz, Reddecliff and Heist (1964b) fed brown trout chloroform-killed and viable A. salmonicida cells, but were unable to induce any significant rise in agglutinating antibody titers. They also induced subclinical furunculosis by injecting sublethal doses of A. salmonicida and studied the subsequent immunity, but they were unsuccessful.

Spence, et al. (1965) fed juvenile coho salmon formalin-killed cells of A. salmonicida. In the first experiment, three groups of fish were held in the laboratory and fed the killed cells for varying

lengths of time. When these fish were artificially infected, the results were not conclusive. No agglutinins were detected in the sera of these fish. In a similar experiment involving 72,000 fish, no protection was induced.

Oral Immunization Against Other Bacteria

Post (1963) attempted to immunize rainbow trout against Aeromonas hydrophila by feeding the fish heat-killed cells of this organism for 272 days. Approximately 27% of the immunized fish survived as compared to 12.5% survival of nonimmunized fish.

Ross and Klontz (1965) were able to immunize rainbow trout against the "etiologic agent of redmouth disease." The trout were fed phenol-killed cells of this bacterium five times a week for the initial two weeks of the experiment, and thereafter, once each week for 10 weeks. The fish (both experimental and control groups) were challenged by intraperitoneal injections of viable cells. The author reported that 90% of the immunized fish and 20% of the nonimmunized fish survived the subsequent infection.

A comprehensive review of the immunological studies on teleost fish has been prepared by Ridgway, Hodgins and Klontz (1966).

EXPERIMENTAL MATERIALS AND METHODS

Culture Media

Furunculosis Agar (Difco Laboratories) was used for routine cultivation and isolation of A. salmonicida.

A. salmonicida cells for preparation of injectable antigens and whole cells for oral immunization tests were cultivated on Mueller-Hinton Agar (Difco Laboratories).

A. salmonicida cells used to prepare alum-precipitated vaccine were grown on Trypticase Soy Broth (Baltimore Biological Laboratories, Inc.).

Cysteine Serum Agar was used for the cultivation of the etiologic agent of bacterial kidney disease. This medium was suggested by Ordal and Earp (1956) for cultivation of this organism. However, human serum (20% by volume) was substituted for human whole blood.

A medium (Cytophaga Agar) described by Pacha and Ordal (1967) was employed for cultivation of Chondrococcus columnaris.

Furunculosis broth was used to grow A. salmonicida cells for artificially infecting coho jacks. The medium was prepared in the laboratory and is composed of 1% tryptone, 0.5% yeast extract, 0.005% L tyrosine, and 0.25% NaCl in distilled water. The medium was sterilized by autoclaving 15 min at 15 lb. pressure at 121°C.

Solutions and Adjuvant

Fresh 1% paraphenylenediamine solution (Eastman Organic Chemicals) was prepared by dissolving 1 g of the crystals in 100 ml of distilled water. The solution was placed in a foil-wrapped bottle and stored at 4°C.

Freund's (complete) adjuvant (Difco Laboratories) required for certain vaccine preparations during this work was stored at 4°C.

Sterile phosphate buffered saline (P. B. S.) was used as the standard diluent in all experiments reported here. The solution was prepared by weighing 8.5 g of NaCl, 0.710 g Na_2HPO_4 and 0.227 g of KH_2PO_4 into a volumetric flask and the total volume brought to 1 liter with distilled water. After the crystals had dissolved, the solution was dispensed into screw cap bottles (200 ml each) and autoclaved at 15 lb. pressure and 121°C for 15 min. The final pH of the P. B. S. was 7.0.

Cultures Used

The A. salmonicida culture used in all experiments, except oral immunization, was isolated in 1962 from an adult fall chinook salmon at the Oxbow Hatchery (Idaho Fish & Game) on the Snake River, and assigned strain no. 5000H. The culture employed in vaccine preparations for oral immunization was the most recent

isolate available from juvenile coho salmon at the Siletz River Salmon Hatchery and was designated strain no. 5006Z.

The Chondrococcus columnaris culture used was isolated from an adult fall chinook salmon at the same Oxbow Hatchery mentioned above.

The bacterial kidney disease organism was provided by personnel of the Fish Commission of Oregon Research Division.

Passive Immunization

Toxicity of Horse Serum to Salmon

Antiserum against A. salmonicida for the passive immunization of adult salmon was prepared in a horse. Since horse serum represents foreign protein, it was first necessary to test the toxicity of this material to salmon. This experiment employed 100 juvenile spring chinook salmon (10 fish/lb.) held in a deep egg-hatching trough divided by perforated aluminum plates into four compartments of equal size. The fish were divided into four equal lots (25 fish each), and one lot placed in each of the four compartments. Each lot was given a number (1-4) and injected intraperitoneally (ip) with either horse serum (Microbiological Associates, Albany, California) or sterile P. B. S. (control). The injection schedule is shown in Table 1.

Table 1. Injection schedule and volume of horse serum or phosphate buffered saline injected intraperitoneally into juvenile spring chinook salmon.

Day	Volume (ml) horse serum injected			Volume (ml) P. B. S. injected
	Lot No. 1	Lot No. 2	Lot No. 3	Lot No. 4
1	0.5	0.75	1.0	1.0
7	0.5	0.75	1.0	1.0
14	0.5	0.75	1.0	1.0
21	0.5	0.75	1.0	1.0

Prior to injection, the fish were anesthetized by placing them in a solution of 16 ml methyl pentynol (Airco Chemical and Plastics Co., New York, N. Y.) per liter of water for 2-5 min or until anesthetized. They were then removed and placed in an egg basket in flowing water. Injection was accomplished before the fish had recovered from the anesthetic. The injection site was selected so that the solution would enter the posterior end of the peritoneal cavity. Fish were observed for distress or death for 1 hr after injection and at daily intervals thereafter.

Antigen for hyperimmunization of a horse was prepared as follows: Mueller-Hinton Agar (Difco) in 8 oz prescription bottles (30 ml/bottle) was inoculated with A. salmonicida strain no. 5000H and incubated at room temperature (21°C-25°C) until maximum growth was obtained. After incubation, 5 ml of sterile P. B. S. was

pipetted into each culture bottle and the growth suspended by gentle agitation with a glass rod. The suspension was decanted into a sterile graduate cylinder, the volume measured and formalin added to a concentration of 0.2%. The formalin-treated suspension was allowed to stand at room temperature for 1 hr and then refrigerated ($4^{\circ}C$) for 24 hr. Sterility was examined by streaking the suspension on Mueller-Hinton Agar and incubating at $21^{\circ}C$ for seven days. Lack of bacterial growth during this period indicated sterility. The number of cells/ml of suspension was determined by use of the Petroff-Hauser bacterial counting chamber. Sterile P. B. S. was used to dilute the bacterial suspension to $3x10^{10}$ cells/ml. For each intravenous (iv) injection of the horse, the desired number of cells were placed in 20 ml of sterile P. B. S., and this volume administered.

To prepare vaccine for intramuscular (im) injection (referred to hereafter as vaccine in adjuvant), Freund's (complete) adjuvant was mixed with an equal volume of the bacterial suspension containing $3x10^{10}$ cells/ml and the mixture emulsified by repeated passage through a 15-gauge needle mounted on a 10 ml syringe.

On May 12, 1966, blood for normal serum was drawn, and hyperimmunization began with an iv injection of the dilute bacterial suspension into the horse's right jugular vein. The injection schedule is shown in Table 2.

Table 2. Injection schedule for hyperimmunization of a horse against Aeromonas salmonicida.

Day	Volume of standard suspension injected	Number of bacteria injected
	Intravenous	
1	0.2 ml plus 19.8 ml P.B.S.	6.0×10^{9}
2	0.4 ml plus 19.6 ml P.B.S.	1.2×10^{10}
3	0.6 ml plus 19.4 ml P.B.S.	1.8×10^{10}
14 days rest		
	Intramuscular	
17	40 ml vaccine and adjuvant	6.0×10^{11}
61	20 ml vaccine and adjuvant	3.0×10^{11}

Some reaction to the vaccine was noted after the first and second injections. Intravenous injections were discontinued after the third injection caused a severe reaction requiring administration of adrenalin and antihistamines to insure survival of the horse. Intramuscular injections of the vaccine in adjuvant were substituted for iv injections. On the 17th day (after 14 days rest), the horse was injected im with 40 ml of vaccine in adjuvant divided between two injection sites on each side of the neck. No reaction was observed from these injections, except for localized swelling. On the 61st day the horse was given a booster im injection consisting of 10 ml of vaccine in adjuvant on each side of the neck.

Antibody formation against A. salmonicida was observed weekly

using a standard agglutinating antibody titration (Kolmer, Spaulding and Robinson, 1951) with antigen prepared according to Spence et al. (1965). After 95 days, agglutinating antibody production had reached a peak, and blood was drawn from the jugular vein into 500 ml centrifuge bottles. The blood was held at room temperature for 24 hr to allow maximum clot retraction; low speed centrifugation (2,000 rpm) was used to separate the serum from the clot. Serum was collected by pipetting and sterilized by Seitz filtration. The serum was then examined for sterility by pipetting 0.2 ml of serum into tubes of thioglycolate broth. The agglutinin titer was determined and the serum stored frozen in 100 ml serum bottles.

Lethal Dose of Aeromonas salmonicida for Coho Salmon

The attempt to passively protect salmon with the anti-A. salmonicida horse serum was preceded by an experiment designed to determine the lethal dose (LD) of A. salmonicida strain no. 5000H for adult salmon. Three circular tanks (6 ft. in diameter) were used to hold 75 coho jacks (approximate length 21 inches), 25 fish in each tank. Each fish in Tank No. 1 was injected with 0.2 ml of a standardized suspension of living A. salmonicida cells. The suspension was prepared as follows: Furunculosis broth was inoculated with A. salmonicida (5000H) and incubated at 18°C for 18 hr. After

incubation, the cells were removed from suspension by centrifugation. The cells were resuspended in sterile P. B. S. and their concentration adjusted to a turbidity which gave a 20% transmittance at 475 mμ (Bausch & Lomb Spectronic 20). Each fish in Tank No. 2 was injected with 0. 2 ml of a 10-fold dilution of the standardized suspension indicated above. Each control fish in Tank No. 3 was injected with 0. 2 ml of sterile P. B. S. All test animals were injected im 0. 5 inch below the dorsal fin. Injections were accomplished while the fish were anesthetized with methyl pentynol 250 ml/1,000 liters water)[2]. All mortalities were observed for gross pathology and examined for the presence of A. salmonicida in external lesions and kidney by inserting a sterile loop into the lesion or organ and streaking onto furunculosis agar. Suspect colonies were examined for gram stain reaction, motility and paraphenylenediamine test. It has been suggested that the coloration or pigmentation of the medium upon which A. salmonicida is grown is the result of tyrosine oxidation. The paraphenylenediamine test is based on the fact that this substance undergoes a color change in the presence of a certain group of enzymes of which tyrosinase is a member (Griffin, Snieszko and Friddle, 1953). After 15 days, all surviving fish were

[2]All adult fish used in this work were anesthetized in this manner.

sacrificed and examined for A. salmonicida in the same manner as were the mortalities.

Passive Immunization of Adult Salmon

The tanks were cleaned, disinfected with Roccal (Sterwin Chemicals, Inc., New York, N. Y.), and stocked with 75 coho jacks to be used for the passive immunization experiment. In this experiment, the 25 fish in Tank No. 1 were injected ip with 5 ml of anti-A. salmonicida horse serum followed immediately with the desired dose of the virulent bacterial cells. The fish in Tank No. 2 also received 5 ml of anti-A. salmonicida horse serum but were not challenged with the cells until 4 hr later. The fish in Tank No. 3 were each given ip injections consisting of 5 ml of normal horse serum and were challenged 4 hr later with the bacterium.

Active Immunization

Direct Injection of Vaccine into Adult Salmon

Thirty adult spring chinook males were divided into five lots of six fish each. Each lot was held in identical deep egg-hatching troughs. The length of each fish was measured and scale samples taken. The scale samples, read by the Fish Commission of Oregon's scale analyst, showed that the fish were four years old. The

vaccine in adjuvant used in this experiment was prepared in the same manner as that used for im injection of the horse. A bacterial suspension in P. B. S. containing $3x10^{10}$ formalin-killed cells/ml was also employed. The fish were anesthetized with methyl pentynol according to the manner previously described. While the animals were still anesthetized, blood for preinjection agglutinin titrations was drawn, and administration of vaccine was accomplished according to the following schedule:

Group No. 1: Two ml vaccine in adjuvant injected im. One ml injected on each side of the fish approximately 1 inch below the dorsal fin.

Group No. 2: Two ml of vaccine in adjuvant injected ip approximately 1 inch behind the pelvic fin.

Group No. 3: One ml of A. salmonicida suspension in P. B. S. in the same manner as Group No. 1.

Group No. 4: One ml of A. salmonicida suspension in P. B. S. injected in the same manner as Group No. 2.

Group No. 5: No vaccine; held as a control lot for normal serum.

Production of agglutinating antibody by the experimental animals was followed employing the same technique used for the horse serum. All blood for analysis was collected from adult salmon by

cardiac puncture of anesthetized fish. The above experiment was repeated with the following changes:

Group No. 1: Received 1 ml of vaccine in adjuvant injected im, 0.5 ml on each side of the fish approximately 1 inch below the dorsal fin.

Group No. 2: Exactly as in Group No. 1 except the dosage was 2.0 ml.

Group No. 3: Exactly as in Group No. 1 except the dosage was 4.0 ml.

Group No. 4: No vaccine; held as a control lot for normal serum.

An attempt was made to repeat this experiment with coho jacks. The immunized fish in this experiment were injected with 1 ml of vaccine in adjuvant on each side approximately 0.5 inch below the dorsal fin.

Oral Administration of Vaccine to Juvenile Salmon

Two circular tanks (6 ft in diameter) were installed at the Siletz River Salmon Hatchery. Annual furunculosis epizootics have occurred in juvenile coho at this hatchery since 1961, providing a natural challenge to the test animals. One thousand juvenile coho (250 fish/lb.) were placed in each of the tanks. One group of

experimental fish was fed the standard Oregon Pellet diet (Hublou, 1963), while the other group received Oregon Pellets containing formalin-killed A. salmonicida cells in the proportion of 7.1×10^{14} cells/kg of food. The bacterial cells were prepared in the same manner as those used for immunization of the horse. However, these cells were lyophilized prior to mixing with the Oregon Pellets. The test group received pellets containing the vaccine on Monday, Wednesday and Friday for 12 weeks, and the normal diet the remaining four days of each week.

A second oral immunization experiment was carried out at the same location. The design of this experiment was as follows: Five 35-gal tanks and one 6-ft circular (320 gal) tank were installed. One hundred fifty juvenile coho were placed in each of the 35-gal tanks and 2,000 were held in the 6-ft circular tank. The fish in tanks 1 through 4 were fed Oregon Pellets containing vaccine. Fish in tanks 5 and 6 received normal Oregon pellets.

The vaccine was prepared as follows (Klontz, 1966): One liter Erlenmeyer flasks containing 300 ml of sterile trypticase soy broth were inoculated with a 36-hr broth culture of A. salmonicida. After 48 hr of incubation at room temperature ($21^{\circ}C$-$25^{\circ}C$), the cells were harvested by centrifugation (5,000 rpm for 30 min). The cells were then resuspended in five times their wet weight in 0.86% saline and mixed thoroughly to insure a uniform suspension. The suspension

was divided into 50 ml batches and the cells disrupted by sonication (Raytheon Model DF101) for a period of 3 hr. The sonicate was centrifuged at 15,000 rpm for 60 min and the supernatant fluid decanted into a flask. An equal volume of 10% potassium alum ($K_2SO_4 \cdot Al_2(SO_4)_3 \cdot 24H_2O$) was added to the solution and thoroughly mixed. Upon addition of potassium alum, a precipitate was formed. Using a Pasteur pipette, NaOH (5N) was added slowly until the precipitate began to go into solution. This point was noted when a drop of NaOH went to the bottom of the suspension. The suspension was centrifuged at 3,000 rpm for 30 min and the supernatant fluid decanted and retained. The precipitate was washed by resuspending in saline and centrifuging (3,000 rpm for 30 min) three times. After final washing, the precipitate was lyophilized and stored in a moisture-proof container at -10°C. To the original supernatant, a drop of NaOH was added. If a precipitate formed, NaOH was added until the precipitate began to dissolve. This material was treated in the same manner as the original precipitate. The lyophilized, precipitated vaccine was incorporated into the Oregon Pellet diet at the rate of 200 mg/1,000 gm of ration. To insure uniform distribution, the vaccine was mixed thoroughly with the dry portion of the diet before the moist portion was added.

The feeding schedule for this experiment was as follows:

Lot No. 1: Fish were fed vaccine-containing pellets daily for 14 days and once weekly thereafter. Feeding of vaccine pellets began on March 7, 1966 and continued until August 1, 1966.

Lot No. 2: Fish were treated exactly as were the fish in Lot No. 1, but feeding of vaccine pellets began 30 days later on April 5, 1966.

Lot No. 3: Same as Lot No. 1, but feeding of vaccine pellets began 60 days later on May 5, 1966.

Lot No. 4: Fish received only the initial 14 feedings of vaccine pellets beginning on April 18, 1966. No additional weekly feedings were administered.

Lot No. 5: Fish received no vaccine pellets. They were fed normal Oregon Pellets throughout the experiment. These fish were the control lot.

Lot No. 6: Fish in this lot were examined weekly in an attempt to detect the presence of subclinical A. salmonicida infection. This lot served as an additional control and received no vaccine.

All fish in this experiment were fed normal Oregon Pellets as a regular diet. The fish were fed both the regular and vaccine-containing diets according to the standard feeding schedule used at this hatchery.

All mortalities were examined for presence of A. salmonicida by culturing kidney inocula on furunculosis agar. All suspect colonies were gram stained, examined for motility and subjected to the paraphenylenediamine test.

Fluorescent Antibody Techniques

Preparation of Antiserum

Rabbits were used to produce antiserum for fluorescent antibody experiments. The method for preparation of vaccine for hyperimmunization of rabbits against A. salmonicida was similar to that used for hyperimmunization of the horse. The vaccine for rabbits was made by mixing two volumes of A. salmonicida suspension containing $3x10^{10}$ cells/ml with one volume of adjuvant. A tissue homogenizer (Virtis) operated at slow to moderate speed was used to emulsify this mixture.

The vaccine in adjuvant was injected into the thigh muscles of three 4 to 6 month old rabbits. Two injections, each consisting of 0.75 ml or a total of 1.5 ml of vaccine corresponding to $3x10^{10}$ bacterial cells, were given to each rabbit (Freund et al., 1948). One rabbit was retained as a source of normal serum, receiving no injections. Seven days after injection of the vaccine, the rabbits were test-bled (2 ml of blood from each rabbit) by ear vein puncture

according to the method outlined by Kolmer et al. (1951). The blood was allowed to clot and then held for 12 hr at 4°C to insure maximum clot retraction. The serum was separated from the cell fraction by centrifugation in a clinical centrifuge. Serum was removed by pipetting, and agglutination tests performed immediately. A. salmonicida antigen for agglutination tests was prepared according to the method described by Spence et al. (1965) and used immediately. Agglutination tests were carried out according to Kolmer et al. (1951) with the exception that greater dilution of the serum was required. Agglutinating antibody titers were performed weekly until the titer was sufficiently high for use in fluorescent antibody techniques. When titers had reached a static level, the rabbits were bled (40 ml of blood from each rabbit) as described above. The serum was harvested and treated in the same manner as described previously for the horse serum. Production of antisera for the kidney disease bacterium and for Chondrococcus columnaris was accomplished in the same manner except that the kidney disease organism was grown on cysteine serum agar, and C. columnaris on Cytophaga Agar.

Fluorescent Antibody Tests

The fluorescent antibody test employed in this study was the indirect method for an unknown antigen as described by Cherry et al.

(1965). Modifications of the technique tried included varying the dilution of antiserum and/or conjugate, the incubation time and temperature. The fluorescein-labeled conjugates used were goat anti-rabbit (Microbiological Associates Inc., Albany, California) and sheep anti-rabbit (The Sylvana Co., Millburn, New Jersey).

Dried tissue powder for adsorption of nonspecific antibody was prepared as follows: The liver, kidney and spleen were removed from juvenile spring chinook which had no known previous history of furunculosis. A Waring blender was used to homogenize 50 g of these tissues with an equal volume of 0.15 M NaCl. The homogenized tissue was placed in a beaker, and four volumes of acetone added, and the mixture stirred. The acetone was then removed by decanting. The tissue was washed by resuspending in saline (0.85%), and then separated by centrifugation (3,000 rpm for 15 min). This process was subsequently repeated three times. The tissue was then suspended in an equal volume of saline, and four volumes of acetone added. After the tissue had settled, the supernatant fluid was decanted and discarded. Four volumes of acetone were again added to the tissue which was subsequently separated from the solvent by means of a Buchner filter. The tissue powder was washed with acetone and dried in the Buchner filter overnight at room temperature. The powder was used immediately to adsorb unwanted

antibodies by adding it to the test serum at the rate of 100 mg/ml. The mixture of serum and tissue powder was allowed to stand for 1 hr; then the powder removed by centrifugation at 18,000 rpm for 30 min in the cold (4°C). The above was repeated and the serum preserved by addition of merthiolate at a concentration of 1:10,000. The serum was stored in a refrigerator and used within five days.

RESULTS

Passive Immunization

Toxicity of Horse Serum to Salmon

Since hyperimmune horse serum was to be used in passive immunity experiments, five lots, each containing 25 juvenile spring chinook were injected ip with varying amounts of normal horse serum in an attempt to determine its toxicity to salmon (Table 2, page 16). The effects resulting from injecting the salmon with horse serum are shown in Table 3.

Table 3. The effects of normal horse serum on the survival of juvenile spring chinook salmon.

Lot number	Total number of fish	Milliliters of serum given per injection	Total number of milliliters serum injected	Number of fish surviving	Percent of fish surviving
1	25	0.5	2.0	22	88
2	25	0.75	3.0	22	88
3	25	1.0	4.0	19	76
4	25	0.0[a]	0.0[b]	25	100

[a] 1.0 ml of P. B. S. administered per injection.

[b] A total of 4.0 ml of P. B. S. was injected.

The results suggest that horse serum is somewhat toxic to juvenile

spring chinook salmon when injected in these quantities (2-4 ml total per fish). More deaths were seen in those groups of fish which received the larger volumes of horse serum (4.0 ml) than those which received the lesser amounts (2.0 and 3.0 ml).

Hyperimmunization of a Horse

Serum for passive immunization of adult salmon was produced by injecting the horse (both iv and im) with A. salmonicida vaccine. The increase in antibody titer against this bacterium was followed by measurement of agglutinating antibody. Figure 1 shows that the agglutinating antibody in the serum had stablized at a titer of 1:5,120 after 30 days. On the 61st day, a booster injection of vaccine in adjuvant was given im. This caused the titer to rise to 1:20,480. On the 95th day, an attempt was made to draw 4 liters of blood from the animal; after 750 ml of blood were collected the veterinarian in attendance advised a two-week rest for the animal as well as a diet supplement. Three days later the horse died from a condition diagnosed as anaphylactic shock. Two hundred fifty ml of serum were harvested from the 750 ml of blood drawn. This anti-A. salmonicida serum had an agglutinating antibody titer of 1:20,480.

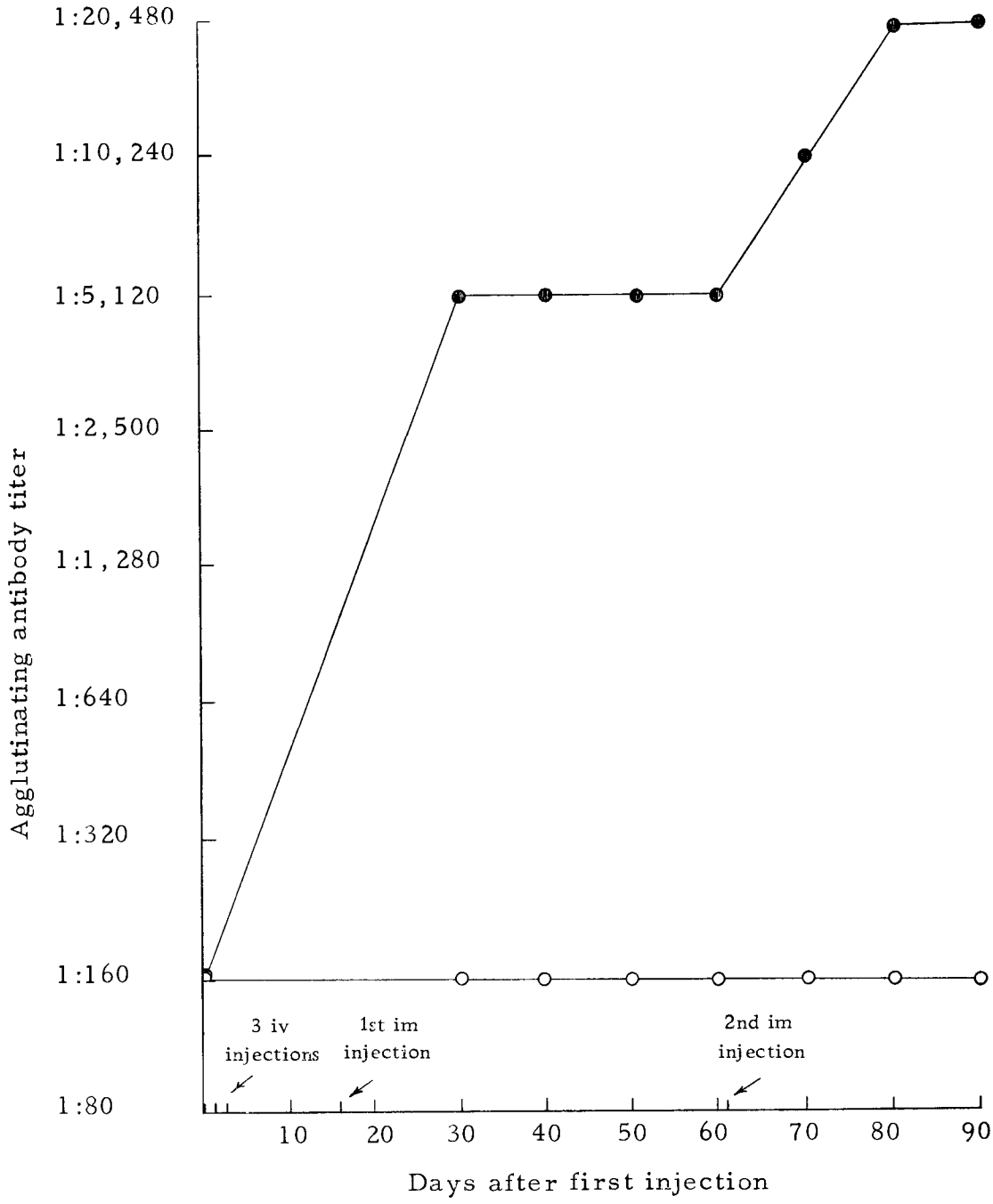

Figure 1. Agglutinating antibody titer of serum from a horse hyperimmunized against Aeromonas salmonicida (●) and compared to the horse serum (o) prior to injection of antigen.

Lethal Dose of Aeromonas salmonicida for Coho Salmon (Jacks)

The lethal dose (LD) of A. salmonicida was established for coho jacks since this knowledge was required to test the passive protection provided by injections of anti-A. salmonicida horse serum.

Table 4. Estimation of lethal dose of Aeromonas salmonicida for coho salmon (jack).

	Number of deaths		
	Lot No. 1[a]	Lot No. 2[a]	Lot No. 3[a]
Days after injection	0.2 ml standard A. salmonicida suspension	0.2 ml 10^{-1} dilution of standard A. salmonicida suspension	0.2 ml P.B.S.
1	0	0	0
2	0	0	0
3	0	0	0
4	1	0	0
5	0	0	0
6	0	0	0
8	6	4	0
9	2	0	0
10	4	0	0
11	2	0	0
Total dead	15[b]	4[c]	0
Percent mortality	60	16	0

[a] Each lot contained 25 fish.

[b] A. salmonicida recovered from all dead fish.

[c] A. salmonicida recovered from only one fish.

Intramuscular injections of 0.2 ml of the standardized A. salmonicida suspension (20% transmittance) produced death in 60% of the

experimental animals within 11 days (Table 4). In the group that received a 10-fold dilution of this suspension, 16% died from furunculosis in the same period. The causative organism could be recovered from only 20% of the dead fish in the latter group. A. salmonicida was not recovered from any of the animals sacrificed at the end of the experimental period (15 days). These results indicate that 0.2 ml of the standardized A. salmonicida suspension constitutes an LD_{60} for these animals.

Passive Immunization of Adult Salmon

The hyperimmune serum which had been harvested from the horse was used in an attempt to passively immunize two groups of coho jacks against A. salmonicida. There was no significant difference in mortality between controls and antibody-treated fish during the 12-day observation period. Autopsy revealed that all the experimental fish had a massive protozoan infection caused by Ceratomyxa shasta. An additional group of coho jacks, being held in the laboratory for another investigation, died at about the same time and manner as did the fish in this experiment. These fish also had a Ceratomyxa shasta infection. At the termination of this experiment, a third group of coho jacks was obtained from the same source and was again found to be infected by this protozoan parasite. These

experiments were terminated because healthy test animals were not available.

Active Immunization

Direct Injection of Vaccine into Adult Salmon

The purpose of these experiments was to determine if adult salmon were able to respond to injected vaccine and to establish which preparation of vaccine and injection route were most effective.

In the first experiment of this series (Table 5), four groups of six adult chinook males were immunized, each in a different manner. All of the fish in this experiment were bled prior to injection, and their sera were tested for agglutinating antibody against A. salmonicida. No agglutinins against this bacterium were observed in any test animals. Twenty days after injection of antigen, one fish from each group was bled and the sera tested to determine if production of A. salmonicida agglutinins had begun. All animals tested were found to have agglutinins (Table 5). After 27 days, all the surviving fish were bled and the serum samples tested for the presence of A. salmonicida agglutinins. The three surviving fish in Group No. 1 (im injection of vaccine in adjuvant) showed titers of 1:640, 1:40 and 1:80. There was no increase in the titer of fish No. 1 which had been tested seven days earlier. Two fish in Group No. 2 survived

Table 5. Differences observed in agglutinating antibody titers among adult spring chinook salmon immunized with two vaccines, each administered by intramuscular and intraperitoneal routes.

Treatment	Fish no.	Recorded agglutinating antibody titer: Prior to injection	20 days after antigen injected	27 days after antigen injected	34 days after antigen injected
Group No. 1 received vaccine in adjuvant (im)	1	0	1:640[a]	1:640	1:1,280
	2	0		1:40	1:160
	3	0		1:80	1:320
	4	0	nd[b]		
	5	0	nd[b]		
	6	0	nd[b]		
Group No. 2 received vaccine in adjuvant (ip)	1	0	1:40[a]	1:160	1:160
	2	0		1:20	1:40
	3	0	nd[b]		
	4	0	nd[b]		
	5	0	nd[b]		
	6	0	nd[b]		
Group No. 3 received vaccine without adjuvant (im)	1	0	1:40[a]	1:160	1:40
	2	0		1:40	1:40
	3	0		No titer	No titer
	4	0	nd[b]		
	5	0	nd[b]		
	6	0	nd[b]		
Group No. 4 received vaccine without adjuvant (ip)	1	0	1:10[a]	1:160	1:320
	2	0		No titer	nd[b]
	3	0	nd[b]		
	4	0	nd[b]		
	5	0	nd[b]		
	6	0	nd[b]		
Group No. 5 received P.B.S. (im and ip)	1	0	No titer[a]	No titer	No titer
	2	0		No titer	No titer
	3	0	nd[b]		
	4	0	nd[b]		
	5	0	nd[b]		
	6	0	nd[b]		

[a] Test bleeding only one fish from each group tested.

[b] No data, fish dead.

for 27 days (ip injection of vaccine in adjuvant). The sera of both these fish showed agglutinating antibody titers; however, the titer of fish no. 1 had increased from 1:40 to 1:160. In Group No. 3 (im injection of vaccine) three fish survived for this period (27 days). Two animals in the group produced antibody (1:40 and 1:160); however, the third did not. Fish No. 1 in this group showed an increase in titer (1:40 to 1:160). There were two surviving fish in Group No. 4 (ip injection of vaccine). The agglutinin titer of fish No. 1 had increased from 1:10 to 1:160, but no agglutinins were detected in the serum from the second survivor. Agglutinins against A. salmonicida were not detected in the sera of two surviving control animals (received injections of P. B. S.).

Thirty-four days after vaccination, all surviving animals were bled and the sera again tested for agglutinins against the antigen. In Group No. 1, the three fish remained, and each showed a considerable increase in agglutinin production. The first animal had increased from 1:640 to 1:1,280, the second from 1:40 to 1:160 and the third from 1:80 to 1:320. Both animals in Group No. 2 which were alive after 27 days survived for 34 days. The first fish in this group did not show an increase in antibody production (1:160); however, the titer of the second animal had doubled (1:20 to 1:40). The three fish remaining in Group No. 3 did not show an increase in

agglutinin production during this period. In fact, the agglutinin titer of the first fish in this group had decreased. This animal was near death, and the serum was completely colorless. The third animal in this group again showed no titer. One of the fish in Group No. 4 had died. This fish did not have an agglutinin titer 27 days after injection. The surviving fish showed increasing antibody titer from 1:160 to 1:320. No agglutinins were detected in the sera of either control fish after 34 days.

In summary, 9 fish survived for 34 days after injection. Eight of these animals were able to produce agglutinins against the antigen injected. Seven of the eight fish which produced antibody showed an increasing titer during the experimental period. All dead fish were examined for A. salmonicida using the standard techniques previously mentioned for isolation and identification of this bacterium; this organism was not isolated from any test fish. The average water temperature during this experiment was 56°F.

Since im injection of vaccine in adjuvant appeared to be the best method of immunizing these fish, this portion of the experiment was repeated. In this test, the amount of vaccine in adjuvant (1.5×10^{10} cells/ml) injected was varied. The fish in Group No. 1 received 1.0 ml, Group No. 2 received 2.0 ml, and Group No. 3 received 4.0 ml of vaccine in adjuvant by the im route. These fish

were all fully ripe males; thus, most died of natural causes. Four fish from Group No. 1 died one day after injection, and all fish in this group had died before the 14th day. In Group No. 2 only one fish survived for more than one day, and it died prior to the 21st day of the experiment. Two fish in Group Nos. 3 and 4 survived for 21 days after injection.

All others died either one or two days after injection. Both surviving fish in Group No. 3 showed the same agglutinating antibody titers (Table 6).

Table 6. Agglutinating antibody response to various doses of vaccine in adjuvant administered by intramuscular injection to adult spring chinoook salmon.

Group	Amount of vaccine injected	Antibody titers at indicated time after vaccination					
		4 days	7 days	11 days	14 days	18 days	21 days
1	2 ml	0	0	1:10	nd[a]		
2	3 ml	0	0	1:20	1:20	1:10	nd[a]
3[b]	4 ml	0	0	1:40	1:40	1:40	1:80
4[b]	0 ml	0	0	0	0	0	0

[a]No data; all fish dead.

[b]Results from two fish which survived for 21 days, both had identical titers.

Even though most of the fish in this experiment died before definite information could be obtained, the results observed suggest

that Group No. 3, which received the largest amount of vaccine in adjuvant, produced the highest titer (1:80). No A. salmonicida could be isolated from any of the fish which died during this experiment.

Another experiment was started in which coho jacks were vaccinated using this technique. The fish were to be artificially infected as soon as agglutinating antibody titers were observed; however, they died from Ceratomyxa shasta infection before detectable antibody developed.

Oral Administration of Vaccine to Juvenile Salmon

Since oral administration of vaccine is the only practical method for immunization of large numbers of juvenile fish, two experiments were performed to determine if this technique could produce protective immunity in juvenile coho salmon.

The first experiment of this series employed whole formalin-killed A. salmonicida cells incorporated in the Oregon Pellet diet (Spence et al., 1965). The antigen-containing pellet diet was fed for 37 days prior to the first death caused by a natural epizootic of A. salmonicida infection. The 1,000 fish which were fed antigen-containing pellets received a total of 7.1×10^{14} cells, assuming that all the diet was consumed. The loss caused by A. salmonicida infection among those fish which had received formalin-killed cells

in their diet was 10.5%; among those that received the normal diet, the loss was 13.7%. This difference was not considered significant in light of the degree of protection desired against the disease. The average water temperature for weekly periods during the experiment is shown in Table 7, along with the minimum and maximum temperatures recorded for the individual weeks. It can be seen that the

Table 7. Minimum, maximum and average water temperatures for weekly (7 day) periods during an attempt to orally immunize juvenile coho salmon using whole cells of Aeromonas salmonicida as antigen.

Weekly period	Average water temperature (°F)	Minimum water temperature (°F)	Maximum water temperature (°F)
4-19-65 to 4-25-65	50.4	48	54
4-26-65 to 5-2-65	51.3	46	58
5-3-65 to 5-9-65	47.2	42	54
5-10-65 to 5-16-65	53.3	48	59
5-17-65 to 5-23-65	51.7	48	57
5-24-65 to 5-30-65[a]	53.7	47	61
5-31-65 to 6-6-65	56.5	49	63
6-7-65 to 6-13-65	57.5	53	64
6-14-65 to 6-20-65	55.7	52	62
6-21-65 to 6-27-65	58.9	50	65
6-28-65 to 7-4-65	61.7	50	69
7-5-65 to 7-9-65[b]	64.9	60	70

[a] The first fish loss attributable to furunculosis was observed during this period.

[b] Five day period.

water temperature rose abruptly during the period of May 24, 1965 to May 30, 1965 and continued to rise thereafter, except during the

period ending on June 20, 1965. It may also be noted that the first death caused by furunculosis was associated with an increase in water temperature.

One year later, oral immunization of juvenile coho was again attempted. In this experiment, an alum-precipitated soluble fraction of A. salmonicida cells was fed to the fish. Three groups of fish received 14-day feedings of the vaccine-containing diet, one group in March, one in April, and one in May. All three groups received one-day feedings of vaccine-containing diet each week after the initial feeding. A fourth group received only the initial 14-day feeding of vaccine-containing diet in April. The other two groups (controls), one consisting of 150 fish and the other of 2,000 fish, received no vaccine. The results of this experiment are shown in Table 8.

Table 8. Results of orally immunizing juvenile coho salmon with an alum-precipitated cell fraction of Aeromonas salmonicida.

Lot no.	Starting no. fish per lot	Starting date	Total vaccine fed each fish in mg	Deaths due to furunculosis Percent	Misc. loss[a] Percent
1	150	3-7-66	57.7	0	3.3
2	150	4-5-66	51.9	0	4.6
3	150	5-5-66	53.5	0.7	4.6
4	150	4-18-66	24.9	0.7	4.0
5	150	No vacc. fed	0.0	22.2	4.0
6	2,000	No vacc. fed	0.0	37.0	11.7

[a] These fish died of cold water disease prior to the furunculosis epizootic.

This table shows that the highest loss from furunculosis in any lot of immunized fish was 0.7% (one fish). In the nonimmunized fish, the loss was 22.2% in Lot No. 5 and 37.0% in Lot No. 6. It can also be seen that each fish in Lot No. 4 received approximately half as much vaccine (24.9 mg) as each fish in the other immunized lots (approximately 54 mg), but did not suffer a significantly higher loss.

The experiment was designed to give some indication of how long before infection the vaccine must be administered to induce protective antibody levels. For this reason, the vaccine administration was begun at various time intervals before the expected epizootic. Immunization of the test animals in Lot No. 4 was begun on April 18, 1966 and completed on May 1, 1966, 27 days prior to the start of the epizootic. The loss of fish in Lot No. 4 was 0.7%, indicating that immunization was accomplished within 27 days of infection.

Figure 2 compares the loss due to furunculosis in the control groups (5 and 6) during the epizootic period with the increase in water temperature. It can be seen that the loss of these fish follows the temperature rise very closely.

Observation of Table 9 shows that losses caused by furunculosis did not begin until the average water temperature had exceeded 55°F. Since the epizootic began during the weekly period ending on May 29, 1966, it could be assumed that any vaccine given after this

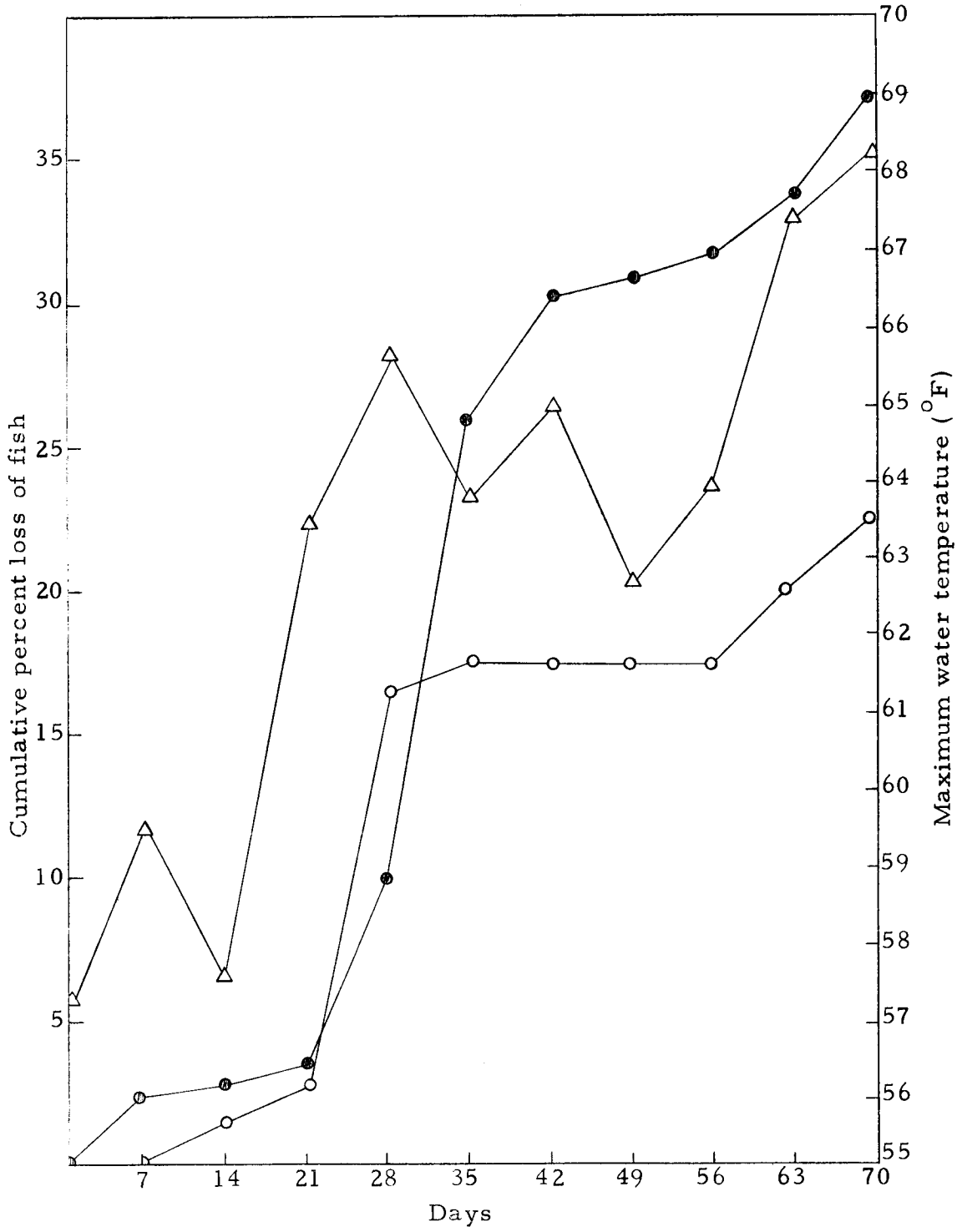

Figure 2. Cumulative percent loss of nonimmunized juvenile coho salmon group 5 (o) and group 6 (●) from furunculosis compared to the average maximum weekly water temperature (△) during the period of May 28-July 31, 1966.

date became less important to practical protection. Therefore, the vaccine primarily responsible for protection against this epizootic was administered at average weekly water temperatures of less than 55.5°F (13°C). It may be further noted that the fish in Lot No. 4

Table 9. Minimum, maximum and average water temperature for weekly (7 day) periods during oral immunization of juvenile coho salmon using an alum-precipitated cell fraction of Aeromonas salmonicida as antigen.

Weekly period	Average water temperature (°F)	Minimum water temperature (°F)	Maximum water temperature (°F)
3-7-66 to 3-13-66	45.5	42	49
3-14-66 to 3-20-66	45.1	42	49
3-21-66 to 3-27-66	47.0	41	51
3-28-66 to 4-3-66	49.3	44	56
4-4-66 to 4-10-66	52.1	47	58
4-11-66 to 4-17-66	49.1	44	55
4-18-66 to 4-24-66	50.1	44	57
4-25-66 to 5-1-66	51.1	45	58
5-2-66 to 5-8-66	54.6	48	61
5-9-66 to 5-15-66	53.1	47	60
5-16-66 to 5-22-66	53.4	46	62
5-23-66 to 5-29-66[a]	55.5	50	64
5-30-66 to 6-5-66	53.7	48	62
6-6-66 to 6-12-66	59.6	55	65
6-13-66 to 6-19-66	62.3	53	72
6-20-66 to 6-26-66	60.3	56	66
6-27-66 to 7-3-66	61.1	55	67
7-4-66 to 7-10-66	61.7	56	69
7-11-66 to 7-17-66	61.1	58	67
7-18-66 to 7-24-66	64.7	60	69
7-25-66 to 7-31-66	64.7	59	70

[a] First fish loss attributable to furunculosis observed during this period.

received vaccine only during the weeks of April 18, 1966 to May 1, 1966, and the average weekly water temperature for these periods was 50.1°F (10.0°C) and 51.1°F (10.5°C), indicating these fish may be immunized at average temperatures as low as 10°C.

Fluorescent Antibody Techniques

Antiserum

In order to obtain antiserum for fluorescent antibody tests, rabbits were hyperimmunized by injecting vaccine (whole cells) in adjuvant into both thigh muscles. This technique worked well and eight days after one injection, the A. salmonicida agglutinating antibody titers in all injected rabbits were 1:5,120, while in the normal rabbit serum no titer could be detected. In 15 days, all rabbits immunized had titers of 1:10,240, and, again, in normal rabbit serum, agglutinating antibody was not detectable. Agglutinating antibody titers were not as high in bacterial kidney disease serum as they were in A. salmonicida serum. One rabbit produced a titer of 1:1,280; the other 1:640. Agglutinating antibody titers in normal serum were not observed. Agglutinating antibody titers for C. columnaris were low (1:40 or less, 35 days after the single injection).

Fluorescent Antibody Tests

Variations of the method suggested by Cherry et al. (1965) for the indirect test for an unknown antigen were tried. It was found that a 1:50 dilution of the goat anti-rabbit conjugate (Microbiological Associates) was required in order to avoid nonspecific fluorescence of A. salmonicida coated with antibody produced in a rabbit. Satisfactory results were obtained in staining smears of A. salmonicida and the kidney disease bacterium by: (1) applying undiluted specific antibacterial serum; (2) washing in buffer for 20 min; and (3) applying 1:50 dilution of labeled anti-rabbit globulin. The anti-rabbit globulin was left on the smear for 15 min at room temperature. Another modification suggested by Klontz (1966) worked well for A. salmonicida but did not for the kidney disease organism. The modification is as follows: Dilute antiserum 1:5 and incubate 30 min at room temperature. Dilute sheep anti-rabbit conjugate 1:5 (Sylvana), rinse 3 min in P. B. S. and 7 min in distilled water.

A. salmonicida, C. columnaris, and the kidney disease bacterium were used to test the specificity of the antiserum produced. The antiserum against A. salmonicida was specific, while that for the kidney disease bacterium reacted with the other bacteria.

A major problem encountered throughout this portion of the study was the unpredictable appearance of nonspecific fluorescence.

An attempt to remove unwanted antibodies by adsorption with a tissue powder preparation of fish kidney, liver and spleen did not alleviate this problem.

DISCUSSION

Passive Immunization

In order to maintain brood fish, most fish hatcheries retain a certain number of juvenile fish which are reared as brood stock. This cannot be done with the salmon, because the life cycle of this fish demands that it migrate to the sea and return to spawn as an adult. In many rivers, the upstream spawning migration of adult salmon is blocked by dams. These fish must then be held in ponds until they reach sexual maturity and are spawned. The propagation of salmon is therefore entirely dependent upon these adults, and in some cases, the number returning is only large enough to maintain that race. The ponds where these fish are held create an environment where disease can be easily transmitted. Under these conditions, passive immunization against certain bacterial diseases becomes feasible.

It has been shown that the serum from fish showing agglutinating antibody against A. salmonicida can be used to passively protect another fish (Spence et al., 1965). With that knowledge, this portion of the investigation was designed to determine if passive immunization could be used to protect returning adult salmon. Large amounts of antiserum required for such an immunization program had to be produced in another animal, and

the horse was selected for this purpose. The possibility that horse serum might be toxic to salmon was investigated. It was found that this serum, when administered in large amounts, was somewhat toxic. The fish used to examine toxicity weighed approximately 0.1 lb., and the smallest amount of serum injected was 0.5 ml. If this weight to volume ratio were projected to a 20 lb. adult fish, the injection (horse serum) would be 100 ml. Since practical passive immunization would require that less than 5 ml of antiserum be injected in an adult salmon, it was not felt toxicity of horse serum would be a problem.

Hyperimmunization of the horse with iv injections of A. salmonicida antigen caused a problem that was expected; it was known that horses are very susceptible to shock reactions of the type encountered. After changing to im injections of vaccine in adjuvant, no immediate reaction was observed. Using im injections for hyperimmunization, the horse produced serum containing high agglutinating antibody titer (1:20,480) against A. salmonicida. When the agglutinin titer had reached this level, blood was drawn from the animal. The experimental plan called for the removal of 4 liters of blood which is well within the prescribed limits for bleeding of horses. After the removal of 750 ml of blood, the veterinarian in attendance noticed signs of anemia in the animal, and advised the

drawing of blood be stopped. Three days later the animal died suddenly. The results of an autopsy indicated the animal died of anaphylaxis. None of the animal's blood was salvaged for serum extraction.

The death of the horse left the investigation with 250 ml of anti-A. salmonicida serum. This serum was used in an attempt to protect adult coho (jacks) against an induced A. salmonicida infection. These fish died as a result of a Ceratomyxa shasta infection before the desired data could be obtained.

Active Immunization

Direct Injection of Vaccine into Adult Salmon

Experiments employing adult salmon are dependent on the availability of surplus fish. These surplus adult fish were males and available when the hatchery personnel removed the fish that were ready for spawning from the holding pond. For these reasons, it was necessary to perform tests on fish that were nearing the end of their life cycle; thus, many died from causes other than infection before the full term of the experiment.

In the first attempt to actively immunize adult salmon, 20 days (incubation period) were allowed after injection before blood was drawn for agglutinating antibody tests. Slightly less than 50% of the test animals survived this 20-day period. These animals were

examined for evidence of infection, and none was found, thus indicating the deaths were caused by other factors. The fact that more than 50% of the fish died less than 20 days after the experiment began is an indication that all were near the end of their life cycle. Despite the debilitated state of the animals, eight out of nine of the remaining fish were able to produce agglutinins, and increasing antibody titers were observed in seven of the eight fish which produced antibody.

The highest titer was observed in Group No. 1 which received im injections of vaccine in adjuvant. All of the surviving fish in this group responded to the treatment. One fish in each of the groups (3 and 4) which received vaccine alone did not respond to the injections of vaccine. The most significant information obtained from these experiments indicates these fish were able to produce antibodies even though near the end of their life cycle. This finding is interesting since there are reports in the literature that indicate adult sockeye salmon (Oncorhynchus nerka) may not be capable of producing isoimmune antibody because of abnormalities in the immune mechanism of these spawning animals. Isoimmunization of these animals was attempted by injecting whole blood into the animals over a period of two to three months (Ridgway and Klontz, 1960; Ridgway et al., 1966).

The limited evidence obtained in this experiment seemed to indicate that the im injection using vaccine in adjuvant was the best method for immunization of these fish. On the basis of this information, a second experiment was initiated in an attempt to determine the proper injection level for vaccine in adjuvant.

Since the fish were near the end of their life cycle, an effort was made to obtain data from more animals by testing for agglutinins on the fourth and seventh days after injection; however, no titers were observed until the 11th day after injection. The fish in this experiment died more rapidly than in the preceding test. Even though the data presented indicate the higher injection level was more effective because of the small number of test animals, the only definite evidence is that these fish can produce agglutinins in response to injected antigens.

A third experiment in this series was designed to determine if the response observed in the previous tests afforded the animals protection against A. salmonicida infection. However, another infection (Ceratomyxa shasta) killed these test animals (coho jacks) before antibodies were produced.

The evidence presented indicates that protection by active immunization could possibly be accomplished in as little as 11 days. If this is so, then protection by passive immunization may not be

needed if runs of salmon could be actively immunized against bacterial infection upon their arrival to holding facilities. While the data presented here only suggest that active immunization of adult salmon is possible, it should encourage further research to determine if they can be protected by this technique.

Oral Administration of Vaccine to Juvenile Salmon

Oral immunization was attempted by feeding juvenile coho salmon whole cells of A. salmonicida that had been killed with formalin. These cells were fed to the fish after being mixed with the normal diet. There was not enough difference in the number of deaths among fish which had received killed cells (10.5%) and those which had not (13.7%) to be of practical value.

An alum-precipitated soluble fraction of A. salmonicida cells was employed in a second attempt to protect fish by means of an orally administered vaccine.

The administration of this cellular fraction produced definite protection. Two lots of fish did not receive vaccine and experienced losses due to furunculosis amounting to 37% and 22.2%. The highest loss in the vaccinated groups was 0.7%.

Water temperatures during the course of the oral immunization experiments were recorded. During both experiments, these

observations showed the epizootics of furunculosis were associated with an increase in water temperature. Very little information concerning the effect of water temperatures on antibody production was obtained, because most of the fish received antigen over a wide range of temperatures, 42°F to 61°F for the first experiment and 41°F to 64°F during the second. One group of fish (Lot No. 4) in the second experiment received vaccine over a somewhat narrower temperature range (44°F to 58°F), and these fish were adequately protected.

There is need for additional studies of the antigenic portion of A. salmonicida cells in order to define and characterize the material responsible for immunity.

Fluorescent Antibody Techniques

The studies presented here were preliminary in nature, and the problem will require a more complete investigation before it can be a useful tool for diagnosis of fish dieases.

SUMMARY AND CONCLUSIONS

1. Horse serum showed slight toxicity for juvenile spring chinook salmon when injected in large doses (2-4 ml). The toxicity observed did not seem high enough to preclude use of hyperimmune horse serum in adult salmon.
2. The intramuscular route used to hyperimmunize a horse was effective, and antiserum of a high agglutinating antibody titer (1:20,480) was produced.
3. All passive immunity experiments had to be abandoned due to the unavailability of experimental animals which were not infected with the protozoan parasite, Ceratomyxa shasta.
4. Adult spring chinook salmon (4 years old) were able to respond to an injected antigen and produced agglutinins for A. salmonicida with titers as high as 1:1,280. These animals were able to respond even though near the end of their life cycle. Seven of the eight fish which produced agglutinins displayed an increasing antibody titer throughout the experiment.
5. The best method of actively immunizing adult spring chinook appeared to be by intramuscular injection of vaccine in adjuvant.

6. Agglutinins against A. salmonicida were observed in the serum of adult spring chinook salmon in as little as 11 days after injection of antigen, the water temperature being 56°F.

7. Juvenile coho salmon were not protected against furunculosis infection by feeding 1,000 fish 7.1×10^{14} formalin-killed cells of A. salmonicida over a 37-day period at water temperatures which ranged from 42°F to 61°F.

8. Definite protection was observed when juvenile coho salmon were fed an alum-precipitated soluble fraction of A. salmonicida cells. The highest loss observed in any group immunized in this manner was 0.7%, whereas losses of 37% and 22.2% were seen in nonimmunized fish.

9. The intramuscular injection of A. salmonicida vaccine in adjuvant employed to hyperimmunize rabbits was effective and produced antiserum with a titer of 1:5,120 in 15 days.

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