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Title: THE ANTIBODY RESPONSE OF JUVENILE COHO SALMON (ONCORHYNCHUS KISUTCH) TO AEROMONAS SALMONICIDA, THE CAUSATIVE AGENT OF FURUNCULOSIS

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Dr. J. L. Fryer

This research was initiated to investigate the effects of various parameters on the immune response of juvenile coho salmon to Aeromonas salmonicida. The antibody response and development of protective immunity in juvenile coho salmon were followed after a single intraperitoneal injection of formalin killed A. salmonicida cells emulsified in Freund's (complete) adjuvant. The first elevated antibody levels and protective immunity were detected four weeks after injection. High antibody titers were observed for a year after injection. Animals as small as 1.2 g were immunologically competent to administration of this antigen preparation. In a representative experiment, 96.7 percent of the animals injected with this preparation produced high levels of antibody.

Selected methods of infection with A. salmonicida were investigated. Variability of culture virulence was demonstrated by injected LD_{50} values, which ranged from a single cell to 10^8 cells.

Endotoxin was extracted from A. salmonicida cells with trichloroacetic acid. This endotoxin was found to be non lethal, but was antigenic to juvenile coho salmon at doses ranging from 1 to 5,000 μg when administered intraperitoneally with and without an aluminum hydroxide carrier. The A. salmonicida endotoxin was lethal to white mice when administered by this same route. LD_{50} values for several preparations ranged from 232 to 536 μg per mouse.

An antibody response to A. salmonicida endotoxin was demonstrated in juvenile coho salmon held at 6.7°C, 12.2°C and 17.8°C. At these temperatures the first elevated antibody levels were observed at four, two and one week, respectively.

The Antibody Response of Juvenile Coho Salmon (*Oncorhynchus kisutch*) to *Aeromonas salmonicida*, the Causative Agent of Furunculosis

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INTRODUCTION

For many years furunculosis disease has plagued trout and salmon fisheries in many countries of the world. The epizootic proportions reached by this disease can on occasion cause great economic loss. Most often the furunculosis outbreaks occur in the crowded conditions of hatcheries. To decrease such high mortality due to this disease, certain prophylactic measures must be instituted. Chemotherapeutic agents such as antibiotics and sulfonamides have been used to efficiently control this disease. However, these agents possess two disadvantages. Often, especially with the sulfonamides, drug resistant strains of this pathogen develop. Second, the effective level of drug is lost from the system soon after administration is terminated.

Another prophylactic measure, which does not possess these disadvantages, consists of the oral administration of an Aeromonas salmonicida vaccine to fish. This treatment is designed to create the immune state in these animals and protect them from the disease for a substantial period of time after vaccination. Concentrated hatchery populations fed a compounded diet lend themselves very well to oral immunization at a modest cost (Frost, 1968). It is proposed that the

vaccine be incorporated into the diet, and the animals fed in accordance with standard hatchery procedures.

In order to vaccinate production stocks of juvenile coho salmon successfully, an understanding of the immune capabilities of these animals to A. salmonicida is necessary. This research attempts to add to our present knowledge of the juvenile coho salmon immune response.

The effects of several parameters on the immune response have been studied. Both whole cell and soluble endotoxin preparations of A. salmonicida antigen were used. One objective of this research was to obtain population immune response data from individual fish for use in predicting potential success of immunization programs. Toward this end, the antibody response of individual coho salmon to a single intraperitoneal injection of A. salmonicida cells emulsified in Freund's (complete) adjuvant was followed by monitoring for agglutinating antibody levels and conferrence of protective immunity.

Water temperature fluctuations existing during the year at most hatcheries could affect the success of fish vaccination. Therefore, the effect of temperature on immune response of juvenile coho salmon was investigated by examining antibody production in these fish held at 6.7°C, 12.2°C and 17.8°C.

Since hatchery vaccination would be performed most often on young fish, the immunological competence and protective value of

oral and parenteral vaccination of very small fish (1.2 g per fish) was investigated.

Observations on the variability of virulence of several A. salmonicida isolates were studied to obtain a better understanding of the infective transfer phenomenon.

LITERATURE REVIEW

The last decade has seen much progress towards an understanding of the evolution of the immune response. The immunological competence of all poiklothermic vertebrates including cyclostomes (hagfish and lamprey), elasmobranchii (guitar fish, sharks and rays), chondrostei (paddle fish), holostei (bowfins and gar), teleostei, dipnoi (lung fish), amphibia and reptilia has been described in recent literature reviews (Clem and Leslie, 1969; Grey, 1969; and Good and Papermaster, 1964).

Immunization of Fish

The prevalence of bacterial, viral and protozoan infections in crowded hatchery situations dictates that prophylactic measures of some type be taken. One such treatment is the administration of a vaccine to create the immune state in animals. The feasibility of immunizing hatchery populations of fish has been demonstrated by the immunological competence of rainbow trout (Salmo gairdnerii) against bacterial antigens (Spence, Fryer and Pilcher, 1965), foreign proteins, such as keyhole limpet hemocyanin (Hodgins, Weiser and Ridgway, 1967) and pathogenic fish viruses such as IPN (Wolf and Quimby, 1969). The response obtained by the latter authors indicates that immunization of fish against viral disease agents using attenuated virus preparations is quite possible.

The literature concerned with immunization of fish has been reviewed recently (Klontz and Anderson, 1970; Snieszko, 1970).

Post (1963, 1966) administered heat killed Aeromonas hydrophila cells to rainbow trout (Salmo gairdnerii) by the oral route and parenterally by the intramuscular (im) and intraperitoneal (ip) route. Although the author indicated that an antibody response was elicited in 50% of the rainbow trout fed the oral vaccine, these individuals were not protected significantly more than control animals when challenged with an ip injected LD_{90} of bacteria. Fish administered the antigen parenterally produced antibody and were protected significantly more than the control group on challenge. The response titers were low and the culture used in this study was avirulent.

Ross and Klontz (1965) administered phenol killed cells of redmouth disease orally to rainbow trout (Salmo gairdnerii). After being fed this vaccine for 70 days, these fish held at 15.5°C were protected from up to a 10 LD_{90} challenge by the ip route. However, Klontz and Anderson (1970) reported that subsequent hatchery immunization with this organism resulted in questionable protection against both parenteral and natural challenge with virulent organisms.

Summerfelt (1966) described the development of protective antibodies by golden shiners (Notemigonus erysoleucas) injected ip with killed Aeromonas liquifaciens cells. Antibody titers were observed two weeks and one injection after the primary antigen administration.

Protection was demonstrated three weeks and two injections after primary antigen administration.

Fujihara (1969) described the oral immunization of rainbow trout against Chondrococcus columnaris, in which the immunized trout suffered a significantly lower mortality than the control group.

Furunculosis Disease and Immunization Against Aeromonas salmonicida

Due to its widespread occurrence in hatcheries, furunculosis is one of the most economically important diseases of trout and salmon. Excellent literature reviews of this disease and its causative agent, A. salmonicida, are available (McCraw, 1952; Herman, 1968).

Aeromonas salmonicida, the etiologic agent of furunculosis, is a short Gram negative non motile rod-shaped bacterium. The infective processes in a furunculosis infection have been described by Klontz (1966). The large losses of hatchery fish have necessitated various prophylactic measures to control this disease. Chemotherapeutic agents operate effectively to limit mortality in hatcheries. However, these treatments are expensive and do not protect fish after treatments cease.

Immunization of fish populations against diseases such as furunculosis would provide an effective prophylactic treatment measure if such a process was reliable, inexpensive, and easily facilitated.

With such large fish populations, only oral immunization complies with the latter two provisions. The reliability question still exists.

The immune response of fish to A. salmonicida has received the attention of several scientists. Smith (1940) demonstrated antibody production of carp and trout held at 10°C to injection of A. salmonicida cells. This worker observed the presence of natural agglutinins (titer = 1:80) in control trout. Gee and Smith (1941) reported a homogeniety of immune response in fish with 26 of 27 carp injected at 20-23°C producing antibodies against A. salmonicida. An antiserum titer of 1:2, 560 was observed in turtles using this antigen.

Duff (1942) recorded the first successful oral immunization of trout against furunculosis. Chloroform killed cells of a virulent A. salmonicida isolate were incorporated into a raw beef-liver (25%) and canned salmon ration (75%) and this diet fed to cutthroat trout (Salmo clarkii) daily or on alternate days for an extended period (greater than 60 days). During immunization, water temperature varied from 2.2°C to 11.6°C. Challenge was accomplished by addition of virulent slant growth of A. salmonicida to fish holding tanks in which water temperature was 19°C. A mortality of 75% in control group compared to a mortality of 25% in the orally immunized group indicated that protective immunity had been gained by the vaccine fed group. Duff (1942) also observed higher antibody levels in the orally immunized fish.

Snieszko and Friddle (1949) could not demonstrate protective immunity in brook trout (Salvelinus fontinalis) orally administered heat killed A. salmonicida cells incorporated into the fish diet at the rate of 0.5 ml cells/kg food. The oral vaccine was administered up until and after the date of challenge. The holding temperature was 12-15°C. Fish challenged with a parenteral dose consisting of 5×10^8 cells, died in equal numbers in the control and orally immunized groups. Oral challenge with 2×10^4 cells caused no mortalities.

Krantz, Reddecliff and Heist (1963, 1964a) demonstrated the production of protective antibodies in brown trout (Salmo trutta) and brook trout (Salvelinus fontinalis) against furunculosis. A high degree of protection was obtained by parenteral injection of formalin killed A. salmonicida cells emulsified in a mineral oil adjuvant, but only low levels of protection were observed after parenteral administration of the formalized cells in saline. Krantz, Reddecliff and Heist (1964b) could show no elevated antibody titers in brown or brook trout orally immunized with chloroform killed or viable A. salmonicida cells and concluded that lack of titers represented lack of protective immunity.

Spence, Fryer and Pilcher (1965) demonstrated the protective nature of immune serum in a passive immunization experiment. A 0.5 ml volume of immune rainbow trout anti A. salmonicida serum (titer greater than 1:320) was injected into juvenile coho salmon weighing 19.3 g each. Significant protective immunity was conferred on

fish administered immune serum, but not on fish administered normal serum or saline. Oral administration of a killed A. salmonicida vaccine to coho conferred no protective immunity and no elevated antibody titers.

Klontz and Anderson (1970) reviewed their efforts to orally immunize trout and salmon with an alum precipitated antigen called FSA that was prepared from the water soluble portion of ultrasonically disintegrated A. salmonicida cells. In a 1965 Washington trial, protective immunity was conferred on brook trout fed 60 μg of FSA for 25 feedings. In 1966, 1967, and 1968, Washington production scale oral immunization programs using juvenile coho salmon were carried out. However, the natural challenge was not of epizootic levels and was insufficient to accurately assess any increase in protective immunity conferred by this treatment.

In 1966 a small production scale oral immunization program was instituted at Fish Commission of Oregon Siletz River Salmon Hatchery (Overholser, 1968) using the FSA antigen as above. Overholser demonstrated protective immunity with 37% and 22.2% furunculosis mortalities occurring in control group and only 0.7% in the immunized group. Overholser also reported the antibody response of adult chinook salmon injected ip and im, with and without Freund's adjuvant. Elevated antibody titers were observed in all groups but many test animals died prematurely after the initial injection.

Frost (1968) reported an oral immunization experiment on a production level of animals. In 1967 at the Siletz River Salmon Hatchery, 520,000 juvenile coho salmon were administered FSA vaccine and 65,000 fish were held as a control group. However, an insufficient natural challenge existed, making it impossible to assess the merits of this immunization treatment.

Frost (1968) observed agglutinating antibody titers in both control and orally immunized fish. However, serum samples from control fish at a hatchery not exposed to a furunculosis epizootic did not contain as high a level of antibody.

Endotoxin from Aeromonas salmonicida

Endotoxins are macromolecules present in the outer membrane of the cell wall of Gram negative bacteria and represent the 0 antigens of the various serotypes or serogroups. A number of methods have been used to extract endotoxins from bacteria (Westphal, Luderitz and Bister, 1952; Nowotny et al., 1963; Crutchley, Marsh and Cameron, 1967; Staub, 1967) and reviews covering the biological activities and composition endotoxins are available (Burrows, 1951; Weyer and Nowotny, 1966). Studies of endotoxins from members of the family Enterobacteriaceae are numerous, but only recently have scientists worked with Pseudomonodaceae toxins. Liu (1961) reported that fresh isolates of Aeromonas bacteria were strongly haemolytic, producing

extracellular toxins that were lethal to mice and elicited haemorrhagic lesions upon injection into skin of rabbit. Meinke and Berk (1970) described a toxic portion of Pseudomonas aeruginosa that had a mouse LD_{50} of 237 μg ip and 105 μg intravenous (iv).

Klontz (1967) reported the extraction of A. salmonicida cells with phenol water, trichloroacetic acid and ether and stated that the former extracts were potent endotoxins based on the results of chick embryo death tests and rabbit pyrogenicity. Ross (personal communication, 1971) stated that these chick embryo LD_{50} values ranged from 0.0019 to 0.0045 μg.

Although these endotoxins cause mortalities in homeothermic vertebrates, similar observations have not been reported with poiklothermic vertebrates. Finstad and Good (1964) reported that lamprey were insensitive to E. coli endotoxin when injected at levels of 0.168 to 168 mg/kg body weight. Berczi, Bertok and Bereznai (1966) tested the toxicity of E. coli endotoxin to a calf, rabbits, dogs, swine, guinea pigs, cats, rats, mice, chickens, frogs, and fish (carp). Death was caused in all except fish, frogs and chickens. Sonnen (1970), in attempts to show toxic effects of endotoxins to frogs, killed the animals (control and endotoxin injected) with elevated temperature.

Wedemeyer, Ross and Smith (1969) demonstrated that endotoxins from Escherichia coli and Aeromonas salmonicida were not toxic (as measured by mortality) to rainbow trout and coho salmon.

Day et al. (1970) suggested that biological responses induced in host by endotoxin are related in part to their potent ability to activate and generate biologically active peptides from the complement system. These workers found that endotoxins were able to consume complement activity in very primitive as well as more highly developed vertebrates. These include lower fishes, amphibians, reptiles, and birds.

Effect of Temperature on the Antibody Response of Fish

In contrast to homeothermic vertebrates, the antibody response of poiklothermic vertebrates is influenced and even controlled by the environmental temperature of the animal. This is quite comprehendible since each type of poiklothermic vertebrate has an optimum environmental temperature at which metabolic functions occur normally. However, in animals held too far above or below this optimal temperature, normal metabolism including synthesis of proteins such as antibody becomes reduced or inhibited.

Early historical literature related to the temperature effect on antibody production in fish has been reviewed (Smith, 1940; Snieszko and Friddle, 1949; and Ridgway, Hodgins and Klontz, 1966).

Hildemann (1962) suggested that temperatures of 20°C or above are generally required to obtain a rapid immune response in fish and amphibians while higher temperatures are required to give comparable results in reptiles.

Smith (1940) demonstrated agglutinating antibody production in response to administration of heat killed A. salmonicida cells to carp, brown trout and rainbow trout held at 10°C. However, the percent response of animals injected was low. Prior to this work, failure had met those attempting immunization at this low temperature. Smith noted the presence of natural anti A. salmonicida agglutinins (titer = 1:80) in control rainbow and brown trout.

Using carp and goldfish injected with sea urchin sperm, Cushing (1942) found that antibodies appeared four days sooner (15 days after injection) in animals held at 28°C than at 14°C.

Ridgway (1962) and Ridgway et al. (1966) demonstrated a primary antibody response in sablefish (Anaplopema fimbria) at 5-8°C within 27 days after antigen administration. This reflects the lower optimal temperature of sablefish.

Fijan and Cvetnic (1966) found no agglutinins in carp administered Brucella antigen when holding temperature ranged between 8 and 15°C, but a strong agglutinating antibody response at temperatures above 15°C.

Avtalion (1969) reported that carp injected im with bovine serum albumin in Freund's adjuvant produced antibodies at 25°C but not at 12°C unless injected at 25°C and held at this temperature for eight days before shifting to 12°C.

Muroga and Egusa (1969) demonstrated antibody production in Japanese eels (Anguilla japonica) injected with Vibrio anguillarum and held at 15°C, but no response was observed at 11°C.

Trump and Hildemann (1970) observed that goldfish (Carassius auratus) produced detectable antibodies against bovine serum albumin at 20, 25, and 30°C with optimal response occurring at 25°C.

Environmental temperature influences the progress of bacterial infections as well as the immune response. Bisset (1946) suggested that normally saprophytic bacteria carried by fish can become lethal parasites if the temperature is raised. Although this worker recognized that fish have more defense mechanism at the higher temperature, he showed that bacterial cultures can become more virulent at higher temperatures. Bisset (1947c) demonstrated that although low levels of bacteria persist in the frog for a longer period of time at 8°C than at 20°C, the increased virulence at the higher temperature overcomes the increased bacterial clearance capacity of the animal, causing infection. The dependence of a disease outbreak on a rise in temperature has been shown (Overholser, 1968; Colgrove and Wood, 1966).

Finn (1970) reviewed the infective process of various infections and the immune response against these fish diseases.

Site of Antibody Synthesis in Teleost Fish

As more information becomes available, evidence implicates a similar type of antibody producing cell in poiklothermic and homeothermic vertebrates. The largest advance in studies of antibody producing cells in poiklothermic vertebrates was made by the adaptation of the rosette test and the Jerne plaque test to cells of these animals. Smith, Potter and Merchant (1967) were the first workers to apply the Jerne plaque technique to teleosts in a study of antibody producing cells of bluegill (Lepomis macrochirus).

Chiller et al. (1969) utilized a rosette test to characterize the morphology of antibody producing cells in the anterior kidney and spleen of rainbow trout (Salmo gairdnerii). The five types of cells capable of forming rosettes with the sheep erythrocyte antigen were lymphocytes, plasma cells, blast-like cells, macrophages and cells resembling eosinophils. The Jerne plaque technique was used to monitor the kinetics of antibody synthesis by lymphoid cells in rainbow trout (Chiller, Hodgins and Weiser, 1969). An increase in plaque forming lymphoid cells (PFC) was observed six days after sheep erythrocyte injection of the fish held at 12°C to 17°C and the peak number of PFC occurred 14 days after injection. Although the majority of PFC were found in the spleen and anterior kidney, PFC in smaller numbers were found in liver, middle and posterior kidney and in

circulatory leucocytes. In this work (Smith et al., 1967; and Chiller, Hodgins and Weiser, 1969) only isologous complement or complement from closely related species hemolyzed the sheep erythrocytes sensitized with bluegill or rainbow trout antibody.

The existence of humoral antibody in fish is a well documented fact. The survival value of these circulating antibodies is questionable with regard to certain types of infections. Recently, immunoglobulins have been detected in the mucus of plaice (Pleuronectes platessa) (Fletcher and Grant, 1969) and of gar (Bradshaw, Richard and Sigel, 1971). If we consider the skin or damage thereof as a major route of entry of infection in poiklothermic animals, then the survival value of mucous antibody becomes obvious.

EXPERIMENTAL MATERIALS AND METHODS

Experimental Facilities

All laboratory work was carried out using the facilities of the Department of Microbiology, Oregon State University. Fish were held at the Oregon State University Fish Disease Laboratory, Corvallis, Oregon; and the Fish Commission of Oregon (FCO) Siletz River Salmon Hatchery.

The fish disease laboratory draws water from a well with a capacity of 150 gal/minute. Ambient water temperature is 12.2°C (54°F) but temperature control equipment allowed experiments to be conducted at temperatures from 3.9°C (39°F) to 23.3°C (74°F).

The four types of animal holding facilities at the fish disease laboratory are explained in Table 1.

Table 1. Aquaria available at Fish Disease Laboratory.

Tank Designation	Size (ft)	Capacity (gal)	Waterflow (gal/min)
circular	4 diameter	180	2
circular	3 diameter	122	2
circular	2 diameter	40	.5
rectangular	2 x 1.5	22	.5

At the Siletz River Salmon Hatchery, fish holding facilities consisted of four and six foot circular tanks for 150 and 1500 fish lots, respectively. The hatchery water source, an adjacent stream, was used in these experiments.

Fish diet consisted of Oregon Moist Pellets (OMP), (Hublou, 1963) or Oregon Test Diet (OTD) (Lee, Roehm and Sinnhuber, 1967). Aeromonas salmonicida vaccine was incorporated into the OTD to be used in oral immunization experiments.

Production of Antigens

Preparation of Aeromonas salmonicida Whole Cell Antigen

Two A. salmonicida isolates were utilized in this research. The A. salmonicida culture isolated from chinook salmon at the South Santiam Hatchery in 1970 was used for investigation of infection methods using the virulent isolate. The cultural methods used with this isolate, designated As SS 70, are described in the section entitled Investigation of Infection Methods Using a Virulent Isolate of A. salmonicida. In all other research described herein, the A. salmonicida culture used was isolated from juvenile coho salmon at the Siletz River Salmon Hatchery during the furunculosis epizootic of 1967 and was designated as As Sil 67.

To maintain uniformity of antigen preparations, a large number of lyophilized stocks of this culture (As Sil 67) were made. In preparation for lyophilization, this isolate was passed twice in juvenile coho salmon and cultured on furunculosis agar (FA[1]) and in furunculosis broth (FB) (Spence et al., 1965). The cells to be lyophilized were grown on FA slants and were harvested in sterile 20% skim milk. The skim milk-bacteria suspension was then dispensed into tubes (0.05 ml/tube containing 3.25×10^9 viable cells/ml) and lyophilized. The stocks were stored at -20°C.

Routine antigen preparations for use in immunization, agglutination tests and challenge work were produced from the lyophilized stocks in the following manner. The lyophilization tube was sterilized in alcohol, and the cells resuspended and cultured in FB for 24 hours at room temperature. The broth culture was used to inoculate FA slants. After incubation for 24 hours at room temperature, the cells were harvested and washed three times with 0.85% saline. A slow centrifugation cycle (1800 RPM for five minutes) was used to sediment any clumps. Culture purity was determined by examining for presence of non motile Gram negative cells and characteristic soluble brown pigmentation on FA. Treatment of the cells from this point varied depending on the intended usage.

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[1] Difco Laboratories, Inc., Detroit, Michigan.

Cell suspensions for immunization and challenge were enumerated using the viable plate count method and/or a Petroff Hausser counting chamber. For immunization, formalin was added to a final concentration of 0.4%. The formalin killed cells were incubated three days at 4°C before sterility was examined in Fluid Thioglycollate Medium (FTM[2]) and on FA plates. If sterile, the cell suspension was mixed 1:1 with Freund's (complete) adjuvant (FCA)[3] and emulsified in glass syringes or a blender. Cells for agglutination tests were not formalin killed.

Preparation of Aeromonas salmonicida Endotoxin and Sonicated Cell Vaccine

A Fermacell Fermentor, Model CF 50[4], was utilized to produce adequate quantities of endotoxin and disrupted cells for vaccine. The A. salmonicida Siletz 67 culture for use in Fermacell inoculation, was previously passed twice through juvenile coho salmon to increase virulence. The lyophilized kidneys of furunculosis infected fish were used as the source of A. salmonicida South Santiam strain (As SS) cells. The lyophilized kidney containing As SS cells was cultured in Brain Heart Infusion Broth (BHIB)[5] and then inoculated into one liter of

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[2]Difco Laboratories, Inc., Detroit, Michigan.

[3]Difco Laboratories, Inc., Detroit, Michigan.

[4]New Brunswick Scientific Co., Inc., New Brunswick, N.J.

[5]Difco Laboratories, Inc., Detroit, Michigan

Trypticase Soy Broth (TSB)[6].

The batch cultures were grown in 20 to 30 liter lots of TSB which was resuspended and sterilized for 60 minutes at 121°C in the fermenter vessel with continuous mixing. Prior to sterilization, 2 ml of silicone antifoam Y 4988[7] were added to each fermacell lot. After cooling the medium to incubation temperature, the fermentor TSB was inoculated with 700-1200 ml of TSB broth culture of A. salmonicida. During incubation, the medium was aerated at a level of 0.25 cubic feet/minute and mixed at an impeller speed of 100 RPM. To replenish carbohydrate supply in the medium, a concentrated filter sterilized (0.45 mμ) dextrose solution was added during incubation of lots one, two and four.

The culture was monitored periodically for purity, pH and cell density by aseptically withdrawing samples from the culture vessel. All samples were found to be pure culture of A. salmonicida.

When cell density was deemed satisfactory, the fermentor culture was collected through the bottom valve and cells harvested in a Sharpless Super Centrifuge Type T1P. Cells were then washed twice in 0.85% saline.

Endotoxin was extracted from both A. salmonicida Siletz 67 and South Santiam 70 cultures. For endotoxin production, the cells were

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[6]Baltimore Biological Laboratory Inc., Baltimore, Md.

[7]Union Carbide Corporation, New York, New York

suspended in distilled water at a level of 1 ml H_2O/200 mg cells. The endotoxin was extracted by a modified method of Staub, 1967. The washed cells were cooled to 4°C and extracted with an equal volume of 0.5 N trichloracetic acid for four hours with mixing. The mixture was then centrifuged and 95% ethanol added to the supernatant to a final ethanol concentration of 68%. After incubation with mixing for 24 hours, the mixture was centrifuged and the pellet washed in 95% ethanol. The precipitate was resuspended in water, dialysed against two 15 liter portions of distilled water, each for 24 hours and filtered through sterile Watman No. 1 filter paper. The endotoxin was then lyophilized and stored at -20°C. Nitrogen analysis (Williams and Chase, 1967) and dry weight determinations were done for the As Sil 67 endotoxin.

For production of disrupted cell vaccine, washed cells were resuspended in 600 ml of saline, frozen and thawed three times and disrupted in 40 ml portions. Each portion was disrupted for four 30 second periods with a Sonifier Cell Disruptor[8]. This treatment yielded near 100% breakage of bacterial cells. Resultant cells of this treatment were designated sonicated cells. These sonicated cells were then formalin killed, incubated several days at 4°C and examined for sterility on FA plates and in FTM. No viable A. salmonicida cells

[8] Heat Systems-Ultrasonics Inc., Plainview, N. Y.

were present. The sonicated cells were then lyophilized and stored at -20°C.

This sonicated cell vaccine was incorporated into OTD for oral immunization and also administered directly by the ip route.

An aluminum hydroxide adsorbant was made according to the procedure of Réthy (1965). The endotoxin was administered to fish with and without this carrier.

Injection and Bleeding Procedures

Fish

Fish were injected with A. salmonicida cells and endotoxin by both the intraperitoneal (ip) and the intramuscular (im) route using a 26 gauge needle. Intraperitoneal injections were made just dorsal to the pelvic fins. Intramuscular injections were made on either side of the dorsal fin. When necessary, fish were anesthetized in a 0.025% methyl pentynol solution prior to injection.

Blood was obtained from juvenile coho salmon by severing the caudal vein just posterior to the adipose fin. Blood to be combined in a pooled sample was dripped into 12 x 75 mm test tubes. Smaller glass tubes were used for blood collection to provide serum samples for individual analysis. These tubes were incubated one hour at room temperature to allow clot retraction, then 12 hours at 4°C before

centrifugation for ten minutes at 2, 000 RPM to separate serum from blood cells.

For small fish (1. 2 to 4. 5 g per fish), heparinized capillary tubes were used for collection of blood. Capillary tubes containing blood samples were centrifuged immediately for five minutes in a hemacrit centrifuge to obtain serum separation.

Rabbit

Anti A. salmonicida serums for serological comparison of cultures and diagnosis of furunculosis were prepared in rabbits. Pre- and post-injection blood was obtained by severing the marginal ear vein and dripping blood into a centrifuge tube. After incubation at 37°C for three hours and 4°C for 12 hours, the serum was separated from the clot by centrifugation. These methods and the ip and iv injection procedures used are outlined (Williams and Chase, 1967).

Mice

Mice were injected with A. salmonicida endotoxin by the ip route using a 1-1/4 inch No. 23 gauge needle (Williams and Chase, 1967).

Serological Procedures

Tube Agglutination

Rabbit sera and pooled salmon sera were analyzed for anti A. salmonicida antibodies using a standard agglutinating antibody titration (Kolmer, Spaulding and Robinson, 1951). Incubation was for three hours at room temperature followed by 12 hours at 4°C. Fresh A. salmonicida antigen adjusted to an optical density (OD) of 0.7 at 520 nm in 0.85% saline was routinely utilized. Titers are reported as the reciprical of the highest dilution showing macroscopic agglutination.

Microtiter Agglutination

All individual coho serum samples were analyzed using the microtiter apparatus (Figure 1)[9]. This apparatus and the analysis of serum by the haemagglutination reaction have been described (Sever, 1962; Witlin, 1966; and Hirata, Grant and Draper, 1969). Vedros and Hill (1966) reported the use of microtiter plates for agglutination tests with Neisseria meningitidis. Disposable V bottom microtiter plates were chosen for use in this work since they gave a clear agglutination end point. Microtiter dilutors of 25 µl or 50 µl size mounted in a multiple dilutor handle and pipets of both the 25 µl and 50 µl capacity were

[9]Cooke Engineering Co., Alexandria, Virginia

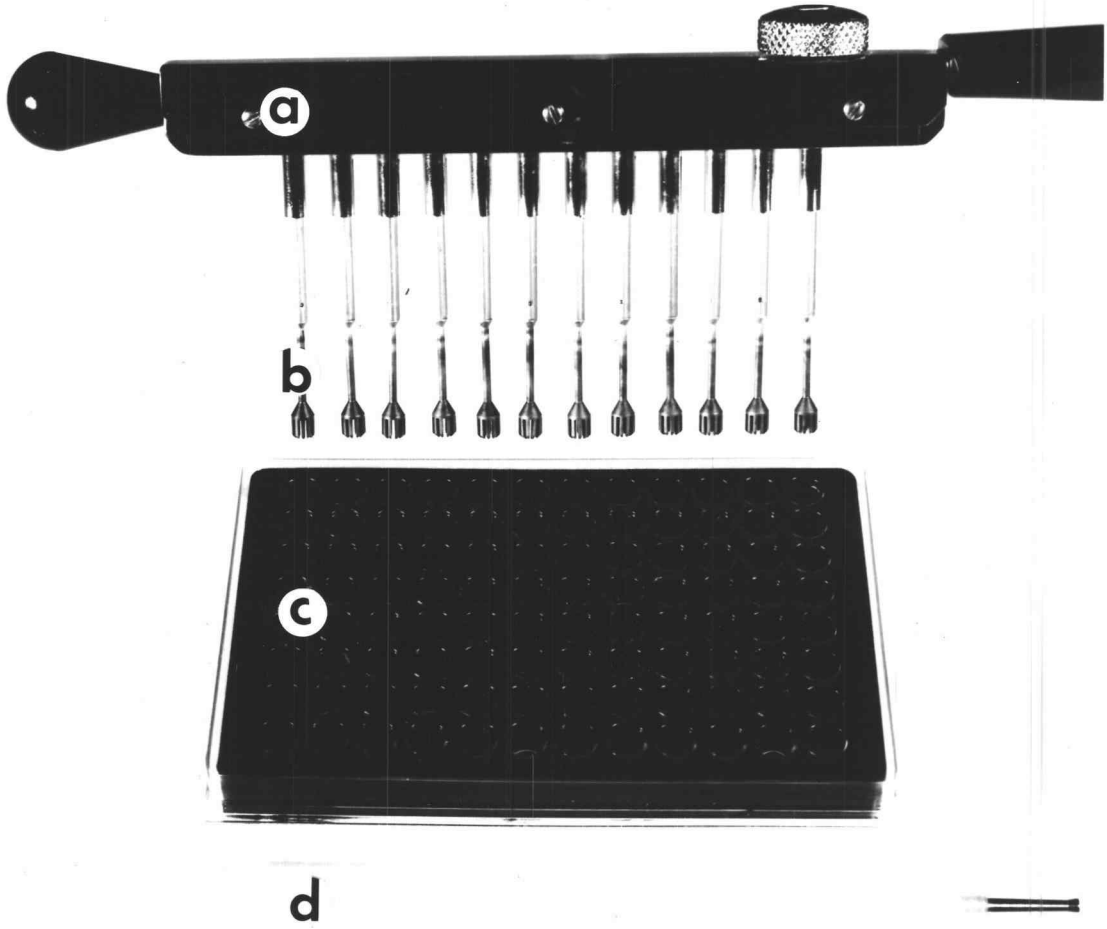

Figure 1. Basic microtiter system consisting of
a. Multiple dilutor handle
b. Dilutors of 50 µl capacity
c. Disposable microtiter plate with V-shaped well bottoms
d. Pipet of 50 µl capacity

utilized in this work. The dilutor and pipet size were determined by the amount of serum available and the initial dilution desired.

The procedure used in microtiter serum analysis was as follows. Either 25 µl or 50 µl of 0.85% saline diluent were added to all wells of a microtiter plate except the first row. These dilutions were made in 0.1 ml pipets by prefilling the pipet with 0.04 or 0.08 ml of saline, followed by addition of serum to the pipet by capillary action to bring total pipet volume to 0.05 or 0.1 ml. The pipet contents were then expelled into the first row wells rendering an initial dilution of 1:5 for both the 25 µl and 50 µl systems, respectively.

Microtiter dilutors previously heated to near incandescence, cooled in air and wet in saline, were gently placed in the first row of wells, allowed to take up their volume of serum dilution, and mixed by rotation of the diluters. Diluters were then placed in the next row of wells and the mixing repeated. This procedure was repeated to the end of the microtiter plate and constituted the serial twofold dilutions. After preparing a titration, the dilutors were blotted, rinsed twice in saline, once in distilled water and then heated to near incandescence before the next usage. A saline control was placed between each set of serum samples. Either 25 or 50 µl of A. salmonicida antigen preparation previously adjusted to an OD of 0.9 at 520 nm was added to each well with the appropriate microtiter pipet. Before reading, the plates were gently mixed, covered and incubated for three hours at

room temperature, then 12 hours at 4°C. A positive agglutination reaction was shown by the uniform dispersal of agglutinated bacterial cells over the bottom of the microtiter well. A negative agglutination reaction was indicated by a button of bacterial cells at the very bottom of the V shaped microtiter plate well (Figure 2).

The agglutinating antibody titers were reported as the reciprocal of the maximum dilution showing the positive agglutination reaction. Reciprocal arithmetic means were calculated for data used in the figures. Reciprocal titer values were converted to logarithmic values to the base ten before statistical analysis. Therefore, the mean values in this instance were geometric.

The precision of the microtiter method with respect to bacterial agglutination tests was determined by multiple analysis of the same serum sample ($titer^{-1} = 640$) obtained 90 days after two injections of 2,000 μg of A. salmonicida endotoxin with aluminum hydroxide. Diluters of 25 and 50 μl size were used for analysis. Tube agglutinations in duplicate and 24 replications using the microtiter system were carried out with this serum.

The mean, standard error, and confidence interval was calculated for each group of replications.

Gel Diffusion

Agar gel precipitin tests were conducted according to Campbell et al. (1964) with a few modifications. Routine qualitative analysis of coho serum utilized 25 x 75 mm microscope slides, with 2.5 ml of 0.85% Ion agar No. 2[10] on each slide. Wells 3 mm in diameter and spaced 4 mm apart were each filled with 0.01 ml of antigen or serum. For routine serum monitoring, a 10^4 µg/ml stock of A. salmonicida endotoxin was used. Slides were incubated in a moist atmosphere at room temperature for three days and observed daily by indirect light for the presence or absence of a precipitin band. Typical precipitin bands formed between immune juvenile coho serum and A. salmonicida endotoxin are shown in Figure 3. The configuration shown in this figure was used in analysis of all individual samples of serum. Three of the four serums tested in Figure 3 show precipitin bands indicative of an immune response.

Challenge Procedures

The dosage of bacteria or endotoxin capable of killing 50% of a population (LD_{50}) was determined by the method of moving averages (Meynell and Meynell, 1965). Fish were challenged by both the ip and im route while mice were challenged by the ip route only. Dilution

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[10] Consolidated Laboratories Inc., Chicago Heights, Ill.

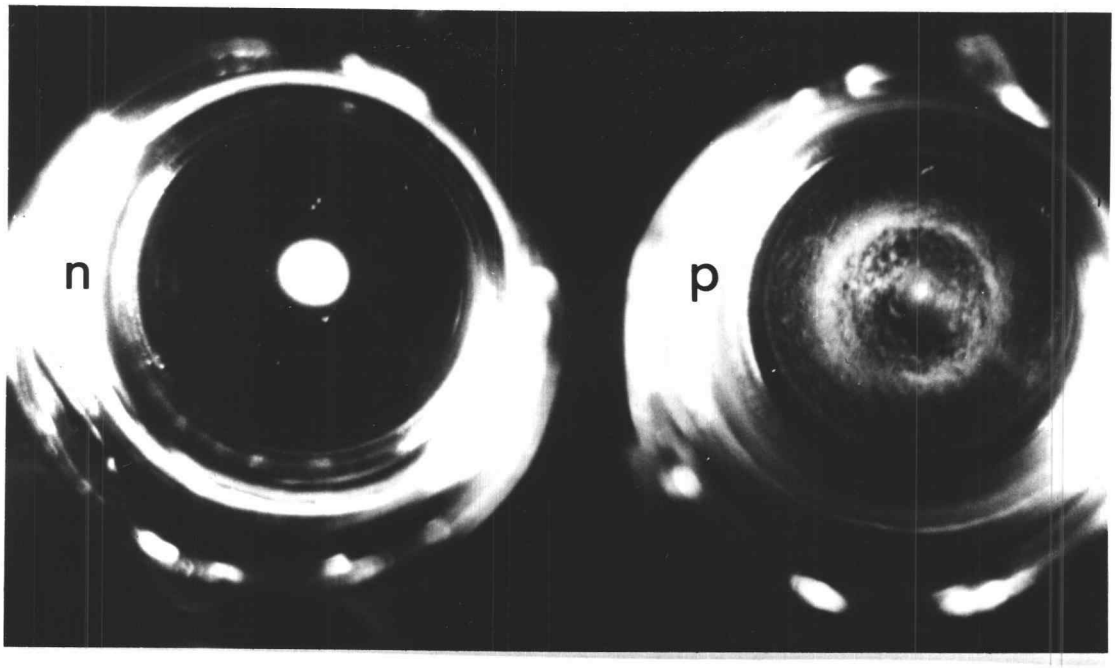

Figure 2. Typical positive and negative Aeromonas salmonicida agglutination reaction using microtiter disposable plates with V bottom wells. p = positive reaction
n = negative reaction

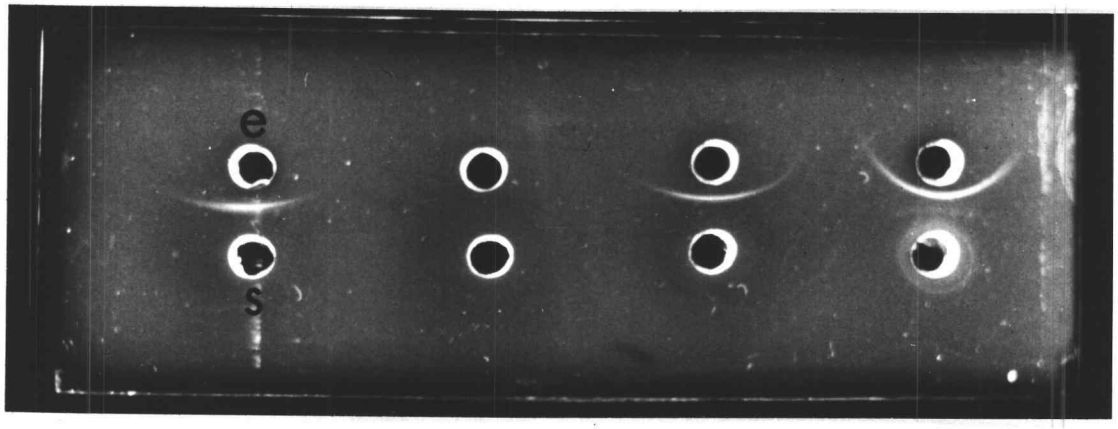

Figure 3. Agar gel precipitin reaction between juvenile coho salmon serum and Aeromonas salmonicida endotoxin.
e = endotoxin well; s = serum well.

factor and sample size per dilution depended on animal type and size, and experimental facilities available.

Fish mortalities were examined for presence of A. salmonicida in the kidney by culturing on FA. Presence of non motile bacteria and brown pigmentation in the media indicated that A. salmonicida was the causative agent of death. Questionable colonies were tested by the slide agglutination test with specific rabbit anti A. salmonicida serum. Only those mortalities from which A. salmonicida was isolated contributed to the LD_{50} values. Other fish challenge methods used are explained in the section on Investigation of Infection Methods Using a Virulent Isolate of A. salmonicida.

Mouse mortalities were examined for gross external and internal pathology. All mortalities contributed to the LD_{50} values during experiments assessing endotoxin toxicity. Saline control injections were done with both mouse and fish challenge experiments.

A typical LD_{50} determination and calculation are presented here as an example. In this experiment, small untreated juvenile coho salmon (weight =2.03g/fish) were injected ip with 0.025 ml of A. salmonicida Siletz 67 cells and held at 12.2°C. These animals were the control fish of the oral immunization experiment. In these particular determinations there were ten animals per dilution and the dilution factor was 0.5 $\log_{10}$. Mortalities were picked and cultured for seven days after injection.

The calculations of LD_{50} values from the infectivity titration data are shown below and in Table 2.

$$f = \frac{0.5 - \overline{m}_1}{\overline{m}_2 - \overline{m}_1} = 0.200$$

$$\log LD_{50} = \log d_o + \log R(f+1) = 7.45 + 0.5(.20+1) = 8.05$$

$$\text{standard error (SE) of } \log LD_{50} = \frac{\log R}{a_3 - a_o} \sqrt{\frac{(1-f)^2 a_o b_o + a_1 b_1 + a_2 b_2 + f^2 a_3 b_3}{n-1}}$$

$= 0.09575$ (Table value from Meynell and Meynell, 1965 for $\sigma_f \times \log_{10} R$)

95% confidence limits for $\log LD_{50}$

$= \log LD_{50} \pm 1.96 \text{ SE } (\log LD_{50}) = 8.05 \pm .18765$

f = proportionate distance between d_1 and d_2 (the log dilutions corresponding to $\overline{m}_1$ and $\overline{m}_2$)

R = dilution factor

a_o and b_o are respectively the numbers of fatal and non fatal injections produced by dose d_o.

n = total number of hosts per dose.

Table 2. An example of an infectivity titration and LD_{50} calculation of *Aeromonas salmonicida* injected juvenile coho salmon using the method of moving averages (Meynell and Meynell, 1965).

Log_{10} Dilution of Culture[a]	No. of Furunculosis Infected Fish Per Total Inoculated (r values)	Mortality	Mean Proportional Mortality ($\overline{m}$)
-2.5	0/10	0	---
-2.0	0/10	0	0.133(= $\overline{m}_o$)
-1.5	4/10	0.4	0.433(= $\overline{m}_1$)
-1.0	9/10	0.9	0.767(= $\overline{m}_2$)
-0.5	10/10	1.0	---

[a]A volume of 0.025 ml of various dilutions of stock culture with a bacterial density = 1.1×10^{11} cells/ml was injected.

Experimental Design

This section will explain the sampling procedures, specifics about the experimental animals and methods used in each experiment. The usage of routine methods will be mentioned, but their full explanation is found in the materials and methods section on general methodology.

Antibody Response of Juvenile Coho Salmon to a Single Intraperitoneal Injection of *Aeromonas salmonicida* Cells Emulsified in Freund's (Complete) Adjuvant

This experiment was conducted to observe the immunological competence of groups of individual fish. Juvenile coho salmon (mean weight = 19.9 g) were obtained from the Alsea Salmon Hatchery, FCO. Two groups of 1500 fish were each placed in separate four foot circular tanks. The water temperature averaged 12.2°C.

One group of fish was injected ip with 0.1 ml of *A. salmonicida* cells emulsified in FCA made as previously described. Each fish was administered 1.75×10^9 *A. salmonicida* cells. This group of fish was the immunized group.

The second group of fish, designated control, was injected ip with 0.1 ml of saline emulsified in FCA.

The immune response of these fish was monitored by bleeding 25 animals from each group every two weeks, beginning on the date

of injection. Individual serum samples were analyzed in duplicate for agglutinating antibody titer using the microtiter technique with 50 µl dilutors. Specific precipitating capacity of individual serum samples was examined on the micro gel diffusion slides.

Means of the reciprocal of duplicate titers were calculated for each fish. Means and standard errors were calculated for each sampling period. Fish with agglutinating antibody titers greater than the upper extreme of the 95% confidence interval for control fish were considered to exhibit an antibody response.

The protective measure of these injections was monitored by LD_{50} determinations conducted on 25 fish from each group every two weeks. A dilution factor of 0.5 $\log_{10}$ and a sample size of five fish per dilution was used. Each fish to be challenged with A. salmonicida cells was injected with 0.05 ml by the im route. Mortalities were collected for 14 days after challenge. Protective indexes were arranged as the ratio of LD_{50} values of immune to control group for each period.

Oral Immunization Experiment

Juvenile coho salmon were vaccinated against furunculosis with a sonicated A. salmonicida vaccine. The protective value of this vaccination was assessed at the Siletz River Salmon Hatchery and the Fish Disease Laboratory. Positive injected controls were inserted

to test the immunological competence of these animals and the ability of these animals to respond immunologically to this sonicated vaccine.

Juvenile coho salmon (mean weight = 0.54 g/fish) were obtained from Trask River Salmon Hatchery, FCO, and divided into four groups at the Fish Disease Laboratory, Corvallis. Water temperature of all groups was 12.2°C. When these animals reached a weight of 1.2 g/fish, the following treatments were initiated.

Group I contained 4,500 fish which were orally administered a sonicated A. salmonicida cell vaccine previously incorporated into OTD. Using an 8% body weight/day feeding schedule, the fish were offered 1000 μg of the sonicated vaccine over a 28 day period. During this 28 day period all other groups were fed OTD without vaccine. The concentration of vaccine in the food was 0.2953 g of vaccine per kilogram of OTD.

Group II consisted of 2,000 juvenile coho salmon, and received OTD without vaccine for the 28 day period.

Group III animals were injected ip with 0.025 ml of A. salmonicida cells emulsified in FCA. Each fish received 2.25×10^9 cells. This treatment was inserted to test the immunological competence of these small animals.

Group IV animals received 500 μg of sonicated A. salmonicida cells in a single 0.025 ml ip injection. This treatment was included to test the animals' ability to respond to this antigen preparation.

Twelve days after completion of the 28 day oral vaccination program the fish were transferred to the Siletz River Salmon Hatchery. A challenge is usually provided by the natural furunculosis epizootic which occurs annually at this location. Mortalities were collected each day and the kidney of each was streaked immediately on a FA plate. Only those fish from which A. salmonicida was isolated, were considered to be infected with furunculosis.

A sufficient number of fish from each group were retained at the Fish Disease Laboratory, Corvallis, to conduct antibody assays of serum and LD_{50} determinations. Six weeks after initiation of treatments, serum was analyzed for antibody titer using the microtiter agglutination test. LD_{50} determinations were conducted using a 10 fish/dilution sample, a 0.5 $\log_{10}$ dilution factor, and ip administration of challenge. Mortalities were collected for seven days after challenge.

Fish injected with cells emulsified in FCA were bled at about monthly intervals for a year after injection. The serum was analyzed for agglutinating antibody titer to observe the duration of the antibody response to a single injection of A. salmonicida cells emulsified in FCA.

Investigation of Infection Methods Using a Virulent Isolate of *Aeromonas salmonicida*

Due to a failure of the natural challenge system, attempts were made to develop a dependable system with a more virulent isolate. This virulent isolate could be utilized in development of challenge methods efficient in assessing the protective degree of oral immunization treatments.

Kidneys were aseptically removed from furunculosis infected chinook salmon obtained from South Santiam Hatchery, FCO. These kidneys were suspended in saline and monitored for presence of *A. salmonicida* by culturing on BHI plates incubated at 18°C for 72 hours.

Of 24 fish examined, 23 yielded gram negative, oxidase positive, non motile bacteria which produced diffusable brown pigment on the culture of the kidney. On basis of these characteristics, the isolates were identified as *A. salmonicida*. BHI spread plates and a cell counting chamber were used to enumerate bacterial cells and assess cell density in infected kidneys.

This isolate was never grown on artificial media, but rather was maintained in kidney tissue of fish by passage through juvenile coho salmon held at 17.8°C. Fish were injected im with furunculosis infected kidney and after death the fresh infected kidney was aseptically removed.

This isolate was compared serologically to the avirulent Siletz 1967 isolate of A. salmonicida. This was done with immune rabbit serum against each isolate using cross adsorption and tube agglutination titrations.

Rabbits were injected with washed formalin killed saline suspensions of A. salmonicida adjusted to an OD of 0.5 at 520 nm (Table 3).

Table 3. Injection schedule for production of rabbit anti Aeromonas salmonicida serum.

Day	Injection (ml)	Injection Route
0	0.1	i.v.
1	0.2	i.v.
2	0.5	i.v.
7	3.0	i.p.
8	1.5	i.v.
9	2.0	i.v.
19	test bleed	
29	bleed	

Antisera were adsorbed with homologous and heterologous antigen preparations. Ten ml of washed formalin killed A. salmonicida cells (concentration of 10 times 0.5 OD) were mixed with 10 ml of 1:10 saline dilution of serum and incubated for two hours at 37°C, then centrifuged. The adsorption of this supernatant was repeated twice more with the final incubation extended to 24 hours at 4°C.

The virulence of several preparations of this isolate was tested. LD_{50} values were determined for several fresh furunculosis infected kidney preparations. LD_{50} determinations were also carried out with

a cultured preparation of this organism. The infected kidney was lyophilized in litmus milk or lyophilization medium[11]. Lyophilized kidney was suspended in BHIB and cultured on BHI plates at 18°C. Cells were harvested and washed three times in phosphate buffered saline, PBS (Williams and Chase, 1967), and then suspended in 0.85% saline for LD_{50} determinations.

The immune response of fish and the protective nature of subsequently produced antibodies to this virulent A. salmonicida isolate were investigated. Juvenile coho salmon (mean weight = 2.8 g/fish) from Trask River Salmon Hatchery were administered a 0.025 ml ip injection of an emulsion of formalin killed A. salmonicida cells and FCA containing 1.2×10^9 cells. Fish were challenged three months after this single antigen injection with cultured preparations of this isolate.

Other selected infection methods were also tried. Juvenile coho salmon were exposed in water and in 0.85% saline containing furunculosis infected kidney tissue. Control fish were held in 0.85% saline without bacterial inoculum. The final concentration of viable A. salmonicida cells in the water or saline was 1000 cells/ml. After a 15 minute exposure period, the 17.8°C water was turned on at a rate of

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[11] Composition of lyophilization medium:
dextran 5%, sucrose 7.5%, monosodium glutamate 1%,
dissolve in water and sterilize.

0.25 gal/min in the rectangular tanks holding 50 fish each. Mortalities in the next 14 day period were examined for the presence of A. salmonicida.

A infection method utilizing contact of healthy with infected fish was attempted. Ten juvenile coho salmon were injected im with a lethal dose of furunculosis infected kidney. Fifty uninjected coho were placed in this 17.8°C rectangular tank with the ten infected fish. The infected fish were allowed to die and decompose in this tank. The uninfected fish were observed for mortalities for 36 days after exposure. Mortalities were examined for presence of A. salmonicida.

Attempts were made to orally infect juvenile coho salmon by direct introduction of 0.1 ml of furunculosis infected kidney (10^6 A. salmonicida cells) into the stomach using a three inch No. 22 needle with a rubber tube covering. Infected kidney was introduced into the stomach of eight fish. Saline was introduced into the stomach of two control animals. Observations were made for one month after treatment and fish were held at 17.8°C.

Effect of Various Dosage Levels of Aeromonas salmonicida Endotoxin on Juvenile Coho Salmon

In order to characterize the effects of various parameters on the immune response, an antigen preparation other than A. salmonicida cells was needed. A. salmonicida endotoxin, the preparation chosen

for testing, was examined for toxicity and antigenicity in fish and mice.

Juvenile coho salmon (mean weight = 7 g per fish) held at 12.2°C were administered two 0.1 ml ip injections of A. salmonicida endotoxin at zero and four week intervals. The endotoxin was administered with and without aluminum hydroxide carrier. Aluminum hydroxide was added at a level of 7.8 mg $Al(OH)_3$ per 20,000 µg of A. salmonicida endotoxin. Dosage of endotoxin ranged from 1 to 5,000 µg (Table 4). The maximum dosage was determined by the solubility of the endotoxin.

Table 4. Dosage of Aeromonas salmonicida endotoxin administered to juvenile coho salmon[a] for determination of toxicity and antigenicity.

Dose per Fish	mg Endotoxin/kg fish
1 µg	.14
10 µg	1.4
100 µg	14
1,000 µg	143
5,000 µg	715
1 µg with $Al(OH_3)$	.14
10 µg with $Al(OH_3)$	1.4
100 µg with $Al(OH_3)$	14
1,000 µg with $Al(OH_3)$	143
4,000 µg with $Al(OH_3)$	572

[a]based on 7g per fish mean weight.

Fish were observed for toxicity and immune response. Mortality was used as the index of toxicity. The immune response was monitored using a 20 fish pooled serum sample and individual serum samples taken from another ten fish. Serum taken eight weeks and 12 weeks after the first injection was analyzed for specific antibody using agglutination and gel diffusion tests. An antibody response was indicated if titers^{-1} were equal to, or greater than, 160.

In the event that the TCA extraction procedure had reduced the toxicity of A. salmonicida endotoxin, another preparation prepared by a mild procedure was utilized. The mildest method available, the use of a culture broth supernatant, was used. Crutchley et al. (1967) previously demonstrated that "free endotoxins" are liberated into the culture supernatants of a wide variety of Gram negative organisms.

The preparation and injection of the A. salmonicida culture supernatant to observe its toxic and antigenic effects on juvenile coho salmon was as follows. Aeromonas salmonicida Siletz 67 was cultured in FB. This broth culture was streaked on FA after 48 hours incubation at 25°C. The contents consisted only of A. salmonicida cells. A streak on FA of this broth after seven days incubation at 25°C showed no growth. The broth culture was then centrifuged (20 minutes at 5, 000 RPM) to remove cellular material and filter sterilized (0. 45 mμ). Sterility was examined and confirmed in FTM and BHI.

A volume of 0.1 ml of this sterile supernatant designated as "free endotoxin" was injected either im or ip into juvenile coho salmon of weight 25.2 g/fish. Twenty fish were injected by each route and 20 control fish were held, but not injected. The fish were held for 22 days at 12.2°C and at this time five fish from each group were bled and the serum analyzed for anti A. salmonicida agglutinating antibody titers.

Toxicity of Aeromonas salmonicida Endotoxin to Mice

The effect of A. salmonicida endotoxin on warm blooded animals was investigated using mice as representative animals. LD_{50} determinations were carried out using 0.5 ml ip injections of endotoxin. A dilution interval of one half and a sample size of five mice per dilution was used. Internal and external gross pathology was observed after injection. Dead mice were removed upon detection.

Effect of Temperature on the Antibody Response of Juvenile Coho Salmon to Aeromonas salmonicida Endotoxin

Temperature affects the rate and extent of immune response in fish. This fact is of importance in hatchery and wild situations where climate and water source cause fluctuations in water temperature. To run reliable oral immunization programs, it is important to know if the fish under question are capable of an immune response at these various temperatures.

The immune response of juvenile coho salmon was followed at 6.7°C, 12.2°C and 17.8°C. Although incomplete, preliminary data was also obtained from an experiment at 3.9°C. Experiments were carried out at each temperature with animals of a mean weight of 22.6 and 6.5 g per fish. Animals were acclimated to temperature for one week prior to antigen administration. Each fish was injected ip with 1,000 μg of A. salmonicida endotoxin in aluminum hydroxide carrier. Control animals were injected only with aluminum hydroxide. Individual serum samples taken weekly from five or ten fish in each group were monitored for agglutinating antibody using the microtiter apparatus. A positive antibody response for the larger 6.5 g fish was indicated by a titer^{-1} of 320 or greater. With the smaller fish, a titer^{-1} of 160 constituted a positive antibody response. These values were greater than any values obtained for control animals of appropriate size.

RESULTS

Antigen Production

Four batch cultures of A. salmonicida cells were produced in the Fermacell Fermentor. The cells from each lot were harvested, washed twice in saline and weighed. The Fermacell yield was quite variable, ranging from 4.8 to 14.2 g of cells/liter (Table 5). Variation in the time of dextrose addition, in pH control, and in the time of harvest led to this variation in yield. The cells were then either extracted or sonicated.

Lot three of Fermacell produced A. salmonicida cells was sonicated and lyophilized. A total of 184 g of wet packed cells produced 25.4 g of lyophilized sonicated cells, a yield of 13.6%. The lyophilized sonicated cells were then either incorporated into OTD or resuspended in saline and used directly for injection.

Lot numbers one, two and four were used for production of endotoxin. A total of 8.6 g of lyophilized endotoxin was extracted from 864 g of wet packed cells. The average yield of dry endotoxin from wet packed cells was about 1.0% of the wet weight. The endotoxin yields of each culture are shown in Table 6. It should be noted that these cells were extracted only once; however, several extractions are possible.

Table 5. Production of Aeromonas salmonicida cells with the Fermacell Fermentor in Trypticase soy broth.

Lot No.	A. salmonicida Culture	Incubation Temperature (°C)	Incubation Time (hours)	Yield (g/l)[c]	Purpose	Comments
1	As Sil 67[a]	23	40	4.8	Endotoxin Extraction	+500 ml 28.5% Dextrose at 24 hours
2	As Sil 67	23.5	30.5	10.0	Endotoxin Extraction	+500 ml 28.5% Dextrose at 13 hours
3	As Sil 67	26	48	9.4	Sonicated Vaccine	----------
4	As SS[b]	20	65	14.2	Endotoxin Extraction	+1000 ml 60% Dextrose at 44 hours

[a] A. salmonicida isolated from coho salmon at Siletz River Salmon Hatchery in 1967.

[b] A. salmonicida isolated from chinook salmon at South Santiam Hatchery in 1970.

[c] Weight of wet packed cells per liter of medium.

A partial analysis of the A. salmonicida Siletz 67 endotoxin indicated its content to be 6.6% water and 2.46% nitrogen by weight.

Table 6. Endotoxin yields resulting from extraction of Aeromonas salmonicida cells with trichloroacetic acid.

Culture	Weight of Wet Packed Cells (g)	Dry Weight of Endotoxin (g)
As Sil 67[a]	427	6.235
As SS[b]	437	2.337

[a] A. salmonicida isolated from coho salmon at Siletz River Salmon Hatchery in 1967.

[b] A. salmonicida isolated from chinook salmon at South Santiam Hatchery in 1970.

Serological Procedures

The microtiter procedure possesses high precision in the analysis of small serum samples for agglutination titer. The precision possessed by the microtiter analysis procedure is shown in Table 7. This data indicates a variation of less than ± one dilution. In addition to the precision of the microtiter method, it very efficiently handles numerous small samples.

The slide agar gel diffusion procedure was useful in monitoring anti serum produced against A. salmonicida cells or endotoxin for specific antibodies. A precipitin band resulted only when undiluted immune serum was used. Although this procedure is capable of

Table 7. Precision of microtiter agglutination test using immune juvenile coho salmon serum and Aeromonas salmonicida Siletz 67[a] cells.

Serum	Equipment	Number of Replications	Mean Titer^{-1}	Standard Error ($\log_{10}$ value)	95% Confidence Interval (Titer^{-1})
Coho salmon	Tube	2	640	0.0000	0
Coho salmon	Microtiter 25 µl dilutors	24	622	0.01954	569 to 680
Coho salmon	Microtiter 50 µl dilutors	24	570	0.03678	483 to 673

[a] A. salmonicida isolated from coho salmon at Siletz River Salmon Hatchery in 1967.

detecting as little as 10 μg of endotoxin, 100 μg was routinely used.

Precipitin bands were formed when antigen preparations other than endotoxin were used (Table 8). However, higher levels of antigen were required for detection of these preparations. FSA (Overholser, 1968) could not be detected in the agar gel diffusion analysis. This observation is probably due to this preparation's lack of antigenicity and solubility.

Table 8. Detection of various Aeromonas salmonicida antigen preparations using agar gel diffusion.

Antigen Preparation	Detectable Level
Endotoxin	10 μg
T.C.A. extract of whole cells (ETOH soluble portion)	100 μg
FSA[a]	not detectable
TCA extracted cells	300 μg

[a] Overholser (1968)
Furunculosis Soluble Antigen

The efficiency of the aluminum hydroxide as a carrier for the endotoxin was shown in adsorption studies. The centrifugation supernatant of endotoxin or endotoxin adsorbed by commercial $Al(OH)_3$ produced detectable precipitin bands at the 10 μg endotoxin level. However, when $Al(OH)_3$ was prepared according to the methods of Rethy (1965), the centrifugation supernatant of the endotoxin adsorbed by this $Al(OH)_3$ produced only a slight precipitin band at the 100 μg level.

Antibody Response of Juvenile Coho Salmon to a Single Intraperitoneal Injection of Aeromonas salmonicida Cells Emulsified in Freund's (Complete) Adjuvant

This experiment to follow the immune response of juvenile coho salmon to an antigen injected in an oil adjuvant was designed to observe the immunological competence of these fish as individuals. Due to its slow continued distribution after injection, this antigen administration has been shown to elicit the best immune response in fish. A uniform immune response from most of the animals is necessary to make an oral immunization procedure function efficiently and reliably.

In this experiment, each fish was injected with an 0.1 ml volume of A. salmonicida emulsified in FCA. Antibody response was monitored from 25 individual serum samples taken every two weeks. LD_{50} values were determined from a different 25-fish lot at these same time intervals. Each serum was analyzed for agglutinating antibody titer and precipitin reaction.

The first antibody response to a single injection of cells in FCA was observed four weeks after administration of the antigen. Table 9 shows that high antibody titers persisted from the fourth week to the last sampling at 14 weeks. Similarly, antibody response was detected by the precipitin reaction in a large percentage of the individual samples beginning at the four week sampling period. The control fish possessed antibody specific for A. salmonicida at $titers^{-1}$ ranging from

20 to 160. These could be natural agglutinins or normally formed antibodies resulting from the ubiquitous nature of A. salmonicida.

Column 3 of Table 9 shows the percent antibody response of the animals tested. These figures were constructed by treating the antibody response as a binomial phenomenon. Any sample with an agglutinating titer^{-1} of 320 or greater was considered to have a positive response; a sample with a titer^{-1} of less than 320 had no response. These criteria were arrived at from statistical analysis of the control titers (Table 12). The percent response ranged from 88 to 100% for the six sampling periods starting four weeks after injection. The overall mean response was 96.6%. This indicates that juvenile coho salmon consist of a relatively homogeneous population with respect to immunological competence to A. salmonicida cellular antigens.

Tables 10 and 11 show the frequency distribution of antibody titers of immunized and control animals.

Table 9 also shows the protective nature of the immune response. LD_{50} ratios (vaccine to control) were greater than one for three of the five sampling periods which gave high antibody titers. Relatively high ratios of 5.0 and 4.0 were observed four and six weeks after injection. Thereafter, the ratios were very low and in one case dropped to 0.6. This cannot be explained unless detection of protection by an im challenge injection is variable and unreliable. Later challenge experiments using the ip route were more predictable.

Table 9. Antibody response[a] of juvenile coho salmon[b] to a single intraperitoneal injection of Aeromonas salmonicida cells emulsified in Freund's (complete) adjuvant.

Time After Injection (wks)	Mean Agglutinating Antibody Titer^{-1}	Percent Antibody Response of the Population[c]	Presence of Precipitating Antibody (Number positive/25 Fish)	Ratio of $\left(\frac{LD_{50} \text{ vaccine fish}}{LD_{50} \text{ control fish}}\right)$
0	42	0	0	
2	38	0	0	1.0
4	10860	96	16	5.0
6	21630	100	23	4.0
8	23000	96	16	1.0
10	26265	88	24	1.6
12	28224	100	22	0.6
14	27706	100	23	---

[a]Temperature = 12.2°C

[b]Mean fish weight = 22.6 g/fish

[c]Percent of 25 fish sample with immune response.

Table 10. Frequency distribution of agglutinating antibody titers of serums from juvenile coho salmon administered a single intraperitoneal injection of Aermonas salmonicida cells emulsified in Freund's (complete) adjuvant and held at 12.2°C.

Agglutinating Antibody Titer^{-1}	Frequency of Titers at Two Week Intervals[a] (weeks after injection)							
	0	2	4	6	8	10	12	14
<20	1							
20	5	22						
30								
40	13	2						
60	2							
80	4	1				2		
120								
160			1		1	1		
240								
320				1	2	1	1	1
480								
640			3	1	2	1	2	1
960			2					1
1280			2		1		2	2
1920								
2560			4	2				2
3840			1		1			
5120			3	5	1	2	1	
7680				1	1			
10240			2	1	2	3	1	
15360			2		1		2	
20480				4	2			1
30720			2		1			2
40960			3	10	10	15	16	14

[a]25 fish per sampling period

Table 11. Frequency distribution of agglutinating antibody titers of serums from juvenile coho salmon administered a single intraperitoneal injection of Freund's (complete) adjuvant and held at 12.2°C.

Agglutinating Antibody Titer^{-1}	Frequency of Titers at Two Week Intervals (weeks after injection)							
	0	2	4	6	8	10	12	14
< 20	1							1
20	5	1	6	4	4	7	2	1
30				1				
40	13	5	4	9	5	8	5	8
60	2			4	1		2	4
80	4	17	14	7	14	10	10	9
120		1					4	
160		1	1		1		2	2
320								

Table 12. The 95 percent confidence interval of agglutinating antibody titers of serums from juvenile coho salmon administered a single intraperitoneal injection of Freund's (complete) adjuvant and held at 12.2°C.

Weeks after Injection	Standard Error (of log mean titer)	95% Confidence Interval of Titer^{-1}
0	0.37389	6.2 to 180.7
2	0.39480	1.2 to 405.5
4	0.31446	12.6 to 215
6	0.20974	17.8 to 118.5
8	0.24594	18.7 to 172.2
10	0.25071	14.0 to 134.8
12	0.24155	23.1 to 206.6
14	0.21104	22.8 to 153.0
Overall	0.24110	17.6 to 155

This experiment showed that a high percentage of animals were capable of an immune response and that these animals would lend themselves well to an immunization program.

Oral Immunization Experiment

In 1970 an oral immunization experiment was conducted at the Fish Disease Laboratory and Siletz River Salmon Hatchery. In this work a sonicated A. salmonicida cell vaccine was used in attempts to vaccinate juvenile coho salmon against furunculosis. Each fish was offered 1,000 μg of vaccine. Positive controls consisted of fish injected with either cells emulsified in FCA or sonicated cells in saline. Fish were transported to Siletz River Salmon Hatchery where a natural furunculosis epizootic usually occurs. Sufficient fish of all groups were retained at the Fish Disease Laboratory for determination of anti- A. salmonicida antibody levels and A. salmonicida LD_{50} values.

In the oral immunization experiment at the Siletz River Salmon Hatchery, mortalities were collected daily beginning April 23, 1970 and continuing through July 30, 1970. Furunculosis was first diagnosed June 11, 1970, but maximum cumulative mortality in a single group due to furunculosis was only 3.7%. As Table 13 shows, the percent mortality due to furunculosis was low in all treatments, indicating that a furunculosis epizootic of sufficient magnitude to offer

Table 13. Furunculosis caused mortalities in juvenile coho salmon treated with various Aeromonas salmonicida vaccines followed by natural challenge at the Siletz River Salmon Hatchery.

Treatment	Sample Size (No. Fish)	No. Mortalities (Total)	No. Fish Infected with Furunculosis	% Mortality Due to Furunculosis
Control (No Vaccine)	1500	100	56	3.73
As[a] Oral Vaccine[b]	1500	107	50	3.34
As[a] Oral Vaccine[b]	1500	50	9	0.60
Control (No Vaccine)	150	3	0	0
As[a] Oral Vaccine[b]	150	3	0	0
Injected As[a] Sonicated Cells	150	7	4	2.67
Injected As[a] Cells + FCA[c]	150	1	1	0.67

[a] Aeromonas salmonicida

[b] Each fish was offered 1000 μg of sonicated A. salmonicida vaccine over a 28 day period.

[c] Freund's (Complete) Adjuvant

challenge and thus adequately assess the protective value of the vaccine was not available.

LD_{50} values and antibody titers were obtained from juvenile coho salmon retained at the Fish Disease Laboratory. The LD_{50} determinations were taken to safeguard the possibility that an effective natural furunculosis challenge would not exist at the Siletz River Salmon Hatchery.

This serological and challenge work conducted as an integral part of this experiment did yield some valuable information. Table 14 shows the LD_{50} dose of A. salmonicida cells and the antibody titer of serum samples taken from each group of fish six to seven weeks after initiation of antigen administration. Animals administered sonicated cells by the oral route showed no elevated LD_{50} or agglutinating antibody titer.

However, the sonicated antigen preparation was effective when administered by the ip route. Elevated antibody titers and an increased LD_{50} were observed in these animals. When A. salmonicida cells were administered in FCA by the ip route, a more intense immune response occurred. This treatment resulted in high antibody titers (mean titer^{-1} = 562) and an elevated LD_{50} value; i.e.,

$$\frac{LD_{50} \text{ vaccine}}{LD_{50} \text{ control}} = 6.27.$$

Table 14. Antibody titers and LD_{50} values of juvenile coho salmon previously exposed to various Aeromonas salmonicida vaccine treatments.

A. salmonicida Vaccine Treatment	Mean[a] Agglutinating Antibody $Titer^{-1}$	LD_{50} (Cells[b])	$\frac{LD_{50}\ Vaccine}{LD_{50}\ Control}$
Control (No Vaccine)	22	1.1×10^8	1.00
Oral Sonicated Vaccine	12	1.6×10^8	1.45
IP Administered Cells + FCA	562	6.9×10^8	6.27[e]
IP Administered Sonicated Cells	199	2.1×10^8	1.91[e]

[a]Sample size = 10 fish/treatment

[b]No. of Aeromonas salmonicida cells

[c]95% Confidence interval of control LD_{50}

$\sigma_f = .19149$

$8.0492 \pm .18765$

7.269×10^7 to 1.725×10^8

[d]Freund's (Complete) Adjuvant

[e]Significantly larger than 1.00 at 95% level

The LD_{50} values obtained in fish immunized with an ip injection of sonicated cells or whole cells with adjuvant are significantly larger than the LD_{50} value in the control fish. This shows that protective immunity was conferred by the parenteral administration of these two antigen preparations.

The last two treatments also show that the fish of this small size (mean weight = 1.2 g/fish) were immunologically competent. This is the first immune response data available using such small animals and is of practical importance since the success of oral immunization programs are dependent upon the immunological competence of fish of this small size.

At about monthly intervals, serum samples were taken from the fish injected with A. salmonicida cells emulsified in FCA. Table 15 shows that high agglutinating antibody titers were maintained during the entire sampling period of one year. The maintenance of these high antibody titers over long periods of time was expected since the presence of adjuvant could be detected in fish autopsied several months after injection. From the samples taken 77 days after injection and thereafter, virtually 100% of the population tested possessed elevated agglutinating antibody titers against A. salmonicida.

Although the efficiency of the oral immunization program could not be assessed, this experiment demonstrated that very small fish (1.2 g/animals) are immunologically competent, as demonstrated by

Table 15. Longevity of antibody response in juvenile coho salmon injected intraperitoneally with Aeromonas salmonicida cells in Freund's (complete) adjuvant and held at 12. 2°C.

Time After Injection (Days)	Number of Fish Bled	Mean Agglutinating Antibody Titer^{-1}	Percent of Fish Showing Antibody Response
0	10	22	0
43	10	562	50
77	10	14624	100
106	5	20480	100
140	5	20480	100
219	5	20480	100
261	5	16384	100
281	5	18432	100
324	5	20480	100
356	5	16896	100

high levels of antibody production and conferrence of protective immunity. The homogeneous immune response of 100% of these animals persisted for a year after administration of cellular antigen in the oil adjuvant. This data indicates that oral immunization is feasible with animals of this size.

Investigation of Infection Methods Using a Virulent Isolate of Aeromonas salmonicida.

These experiments were carried out in search of a laboratory challenge procedure to assess oral immunization experiments. This was necessitated since a natural challenge in the form of a furunculosis epizootic could not be relied upon to assess the protective value of oral immunization. Therefore, several other infection methods were investigated using a more virulent A. salmonicida culture.

Aeromonas salmonicida was cultured from 23 of the 24 South Santiam chinook kidney suspensions examined. Kidney suspensions from eight of the furunculosis infected fish were pooled and reinjected into juvenile coho salmon. Using cross adsorption and tube agglutination tests, A. salmonicida strains As Sil 67 (avirulent) and As SS (virulent) were shown to be serologically identical (Table 16). LD_{50} values were determined in juvenile coho salmon for the bacteria in kidneys harvested from A. salmonicida injected juvenile coho salmon. Table 17 indicates the very low LD_{50} values of A. salmonicida cells

in the kidney suspension. At 17.8°C these LD_{50} values approached a single cell; however, this value rose to 75 cells at 12.2°C. For comparison purposes, the LD_{50} of the avirulent A. salmonicida Siletz 67 cultured isolate was 1.1×10^8 cells. The LD_{50} value at 17.8°C of the South Santiam A. salmonicida isolate rose to 348 cells after it had been transferred four times on artificial medium.

Table 16. Cross agglutination and adsorption titers^{-1} of rabbit antisera against As Sil 67[a] and As SS[b] strains of Aeromonas salmonicida.

Antiserum	Adsorbing Strain	Antigen	
		As Sil 67	As SS
As Sil 67	Saline	3840	2560
	As Sil 67	< 20	<20
	As SS	< 20	<20
As SS	Saline	5120	5120
	As SS	< 20	<20
	As Sil 67	< 20	<20

[a]Aeromonas salmonicida isolated from coho salmon at Siletz River Salmon Hatchery in 1967.

[b]Aeromonas salmonicida isolated from chinook salmon at South Santiam Hatchery in 1970.

A cultured A. salmonicida South Santiam preparation was used in challenging fish previously injected with a formalin killed sample of this isolate emulsified in FCA. These fish possessed uniformly high agglutinating antibody levels. The mean titer^{-1} of the five fish tested was 23030.

Table 17. LD_{50} values of Aeromonas salmonicida (South Santiam Strain) injected parenterally into juvenile coho salmon.

Preparation of A. salmonicida South Santiam	Water Temp. (°C)	LD_{50} (No. of Cells)	Standard Error (of log LD_{50})	Injection Route
Kidney suspension[a]	17.8	1.5	0.3965	im
Kidney suspension[a]	17.8	0.6	0.1995	im
Kidney suspension[a]	17.8	2.6	0.2450	im
Kidney suspension[a]	12.2	75	0.3012	im
Cultured As SS[b]	17.8	348	0.5652	ip

[a]Saline suspension of A. salmonicida infected juvenile coho salmon kidney.

[b]After two transfers on furunculosis medium.

Table 18 shows that fish injected with this antigen preparation showed significant protective immunity against an ip administered challenge of A. salmonicida. In the two trials shown in Table 18, there were significantly more mortalities in the control than the immunized group when data was analyzed using chi square. It should be noted that LD_{50} values by the im route were attempted but showed no difference between the immunized and nonimmunized groups. It was demonstrated previously, however, that this route of injection was unreliable in detecting the immune state on occasion.

Table 19 shows that the two contact methods of infection used were not effective. The exposure of 50 untreated fish to 10 infected fish at 17.8°C resulted in only an 8% mortality due to furunculosis in

the untreated fish. Length of exposure time was one week.

Table 18. Challenge of vaccinated[a] and untreated[b] juvenile coho salmon with an intraperitoneal injection of cultured Aeromonas salmonicida (South Santiam) cells.

Sample Size/group (No. of Fish)	Number of Viable cells Injected	Percent Mortality Due to Furunculosis Control	Immune	Chi Square Value
15	1470	40	0	10.18[c]
20	2700	70	15	5.2[d]

[a]Injected ip with formalin killed A. salmonicida (South Santiam) cells emulsified in Freund's (complete) adjuvant.

[b]Injected ip with saline emulsified in Freund's (complete) adjuvant

[c]$\chi^2 = 10.18$ $P(\chi^2 < 6.63; 1\ df) = 0.99$

[d]$\chi^2 = 5.2$ $P(\chi^2 < 3.84; 1\ df) = 0.95$

Table 19. Methods of infecting juvenile coho salmon with Aeromonas salmonicida[b] infected coho kidney tissue.

Method of Infection with A. salmonicida	Number of Cells Administered	Percent Mortality
Im injection	1.5	50
Contact with infected fish for one week	---	8
Contact with bacteria in water for 15 minutes.	10^3/ml water	0
Contact with bacteria in saline for 15 minutes.	10^3/ml saline	0
Oral injection in stomach	10^6	0

[a]at 17.8°C

[b]A. salmonicida South Santiam isolated from chinook salmon at South Santiam Hatchery in 1970.

No mortalities occurred when juvenile coho salmon were exposed for 15 minutes to an A. salmonicida cell density of 1000/ml in saline or water. This indicates that contact of healthy fish with A. salmonicida cells or to furunculosis infected fish under the conditions of these experiments does not necessarily lead to transfer of the disease.

The direct introduction of 10^6 viable A. salmonicida bacteria (in furunculosis infected coho kidney tissue) into the stomach of coho also resulted in no mortalities. The strain used was highly virulent with an LD_{50} by im injection of about one cell.

This data illustrates some of the problems which exist in developing a challenge for furunculosis. The route of transmission of this disease can also be questioned since neither direct contact to the exterior of the fish nor the introduction into the stomach of virulent organisms caused a high incidence of mortality under the conditions of this test.

Effect of Various Dosage Levels of Aeromonas salmonicida Endotoxin on Juvenile Coho Salmon

Antigen preparations of A. salmonicida other than those of cellular type were to be examined for antigenicity, ease of handling and toxicity to fish. Eventually the most potent antigen preparation could be utilized in vaccine production. The preparation examined here

was the TCA extract of A. salmonicida, an endotoxin.

The toxicity and antigenicity of A. salmonicida endotoxin to juvenile coho salmon were determined. This was necessary to assess the value of this antigen preparation for further work in characterizing the immunological capabilities of these fish. In this experiment, juvenile coho salmon were injected ip with levels of A. salmonicida endotoxin ranging from 1 to 5,000 μg. Antibody response was monitored from a 20 fish pooled serum sample and individual serum samples from ten other fish.

No mortalities resulted from the ip injections of A. salmonicida endotoxin into juvenile coho salmon held at 12.2°C (Table 20). This apparent lack of toxicity to fish has previously been reported (Berczi, Bertok and Bereznai, 1966; Wedemeyer, Ross and Smith, 1968).

These fish did respond immunologically to the injection of endotoxin at all levels administered. Table 20 shows the antibody response of fish bled eight weeks after the first injection. Elevated antibody titers were observed in fish injected with endotoxin levels ranging from 1 to 5,000 μg. Although the response appears greater with the aluminum hydroxide carrier, the difference is not statistically significant except in the case of the 100 μg dose.

Titers of fish bled 12 weeks after the first injection were elevated at most endotoxin levels (Table 21), but were lower than those

Table 20. Antigenicity and toxicity of various levels of Aeromonas salmonicida endotoxin to juvenile coho salmon at 12.2°C as measured eight weeks after the first injection.

Dose of Endotoxin per Fish	Pooled Samples[a]		Individual Samples[b]		Percent Mortality
	Agglut. Ab.[c] Titer^{-1}	Presence of Ppt. Ab.[d]	Mean Agglut. Ab.[c] Titer^{-1}	Presence of Ppt. Ab.[d]	
0	120	-	33	0/10	0
1μgm	640	-	336	2/10	0
10μgm	960	-	488	3/10	0
100μgm	960	+	288	0/10	0
1000μgm	1280	+	360	1/10	0
5000μgm	1280	-	204	0/10	0
$Al(OH)_3$	160	-	36	0/10	0
1μgm + $Al(OH)_3$	960	+	1426	2/10	0
10μgm + $Al(OH)_3$	1720	+	980	0/10	0
100μgm + $Al(OH)_3$	1720	+	3376	2/10	0
1000μgm + $Al(OH)_3$	2560	+	1468	1/10	0
4000μgm + $Al(OH)_3$	1280	-	1228	1/10	0

[a]20 fish/pooled sample

[b]10 fish/group. These are separate animals from those used in the pooled sample.

[c]Agglutinating antibody titer

[d]Precipitating antibody

Table 21. Antibody response of juvenile coho salmon to various levels of Aeromonas salmonicida endotoxin 12 weeks after the first injection.

Dose of Endotoxin per Fish	Pooled Samples[a]		Individual Samples[b]	
	Agglut. Ab.[c] Titer^{-1}	Presence of Ppt. Ab.[d]	Mean Agglut. Ab.[c] Titer^{-1}	Presence of Ppt. Ab.[d]
0	160	-	60	0/10
1μgm	480	-	324	0/10
10μgm	1280	-	256	0/10
100μgm	640	-	496	0/10
1000μgm	640	-	1093	0/10
5000μgm	640	-	296	0/10
$Al(OH)_3$	80	-	32	0/10
1μgm + $Al(OH)_3$	640	-	92	0/10
10μgm + $Al(OH)_3$	1280	-	302	1/10
100μgm + $Al(OH)_3$	960	-	720	0/10
1000μgm + $Al(OH)_3$	960	-	164	0/10
4000μgm + $Al(OH)_3$	640	-	548	0/10

[a] 20 fish

[b] 10 fish/group

[c] Agglutinating antibody titer

[d] Precipitating antibody

obtained for the previous sampling period (Table 20).

The percent response of each group at the two sampling periods is shown in Table 22. The overall response for the eight and twelve week samples was 69 and 55 percent, respectively. These values are lower than those obtained from injection of whole cells in FCA. The percent response data indicates that a better response did not necessarily result from injection of higher endotoxin levels.

The number of fish with a positive precipitin reaction (Table 20 and 21) did not parallel the percent response using the agglutination test (Table 23). Generally, an agglutinating titer^{-1} of 1280 was necessary before precipitin reactions could be detected. The much higher percent response obtained using the agglutination reaction indicates the higher sensitivity of this method.

The antibody response of juvenile coho salmon to all levels of A. salmonicida endotoxin from 1 to 5,000 μg suggests that this antigen preparation is ideal for immune response studies using these animals. This preparation may also be useful as a vaccine since it is very antigenic to these fish at levels as low as 1 μg.

To answer the question about the reduction of toxicity of A. salmonicida endotoxin by the TCA extraction procedure, a "free endotoxin" preparation consisting of a sterile A. salmonicida FB culture supernatant was tested for toxicity and antigenicity. A volume of 0.1 ml of this supernatant was injected ip into each animal of a 20

Table 22. Percent antibody response[a] of juvenile coho salmon[b] eight and twelve weeks after two injections of Aeromonas salmonicida endotoxin.

Aeromonas salmonicida Endotoxin Dose/Fish	Percent Antibody Response 8 weeks after first injection	Percent Antibody Response 12 weeks after first injection
0	0	10
1 μg	50	70
10 μg	60	60
100 μg	80	60
1000 μg	80	80
5000 μg	60	50
Average	66	64
$Al(OH)_3$	0	0
1 μg + $Al(OH)_3$	60	20
10 μg + $Al(OH)_3$	90	45
100 μg + $Al(OH)_3$	80	50
1000 μg + $Al(OH)_3$	80	30
4000 μg + $Al(OH)_3$	50	90
Average	72	47
Overall Average of Antigen Injected Fish	69	55

[a]Percent of ten fish samples with agglutinating antibody titer^{-1} greater than 160.

[b]Holding temperature = 12.2°C.

fish group, and the same volume injected ip into each individual of another 20 fish group. Twenty uninjected controls were held. Serum samples were taken from five fish from each group and analyzed for agglutinating antibody titer.

No mortalities were observed during the 22 day holding period after injection. The agglutinating antibody titers^{-1} of the im injected fish were 5,120, 5,120, 640, 320 and 320. Titers^{-1} of the ip injected fish were 640, 1,280, 640, 1,280 and 320. Titers^{-1} of the uninjected fish were 40, 40, 40, 80, and 80.

Although this free endotoxin was not toxic to juvenile coho salmon held at 12.2°C, all of the injected animals tested demonstrated a positive antibody response to this preparation. This shows that even A. salmonicida endotoxin procured by this mild procedure was non toxic to these fish and indicated that the non lethal effect of the TCA endotoxin to fish was not due to the endotoxin extraction procedure.

Toxicity of Aeromonas salmonicida Endotoxin to Mice

Endotoxins of Enterobacteriaceae are toxic to warm blooded animals such as mice, but do not seem to affect cold blooded animals such as frogs or fish. In order to examine the effects of a trichloroacetic acid extract of A. salmonicida on warm blooded animals, mice were injected ip with various levels of this endotoxin.

In contrast to results obtained with coho salmon, the A. salmonicida endotoxin was very lethal to mice. Table 23 shows the LD_{50} values obtained by the administration to mice of endotoxins from the A. salmonicida isolates As Sil 67 and As SS 70. This toxicity is comparable to endotoxins of Escherichia coli for the same animals as reported by Berczi et al. (1966). Aeromonas salmonicida endotoxins are also quite toxic to chick embryos (Ross, 1971).

Mice injected with endotoxin showed reduced activity two hours after injection and by ten hours post injection continued to show this symptom plus a waddling gate and closed eyes. Survivors after 24 hours were inactive with ruffled hair and closed eyes. Diarrhea and loss of bladder control was observed in mortalities. Internal examination revealed occasional swollen intestines and hemorrhaged spots on the lungs. Similar symptoms to these have been reported for a toxic portion of Pseudomonas aeruginosa (Meinke and Berk, 1970).

An animal which survived an injection of 1 mg but showed the symptoms previously mentioned was administered a second 1 mg dose with no adverse symptoms. This former response was characteristic of animals possessing anti-toxin antibodies.

These LD_{50} determinations show that the A. salmonicida endotoxin was toxic to mice, a typical characteristic of Enterobacteriaceae endotoxins. This data together with other information presented earlier suggests that this is a typical endotoxin.

Table 23. LD_{50} values of Aeromonas salmonicida endotoxin administered intraperitoneally to Swiss Webster female mice.

Endotoxin	LD_{50} µg	LD_{50} mg/kg[c]	95% Confidence Interval (µg)	Mean Mouse Weight (g)
As Sil[a]	324	17.6	226 to 465	18.42 ± 2.78
As Sil	232	11.7	152 to 358	20.17 ± 1.32
As SS[b]	536	21.7	385 to 747	24.75 ± 2.29

[a]A. salmonicida isolated from coho salmon at Siletz River Salmon Hatchery in 1967.

[b]A. salmonicida isolated from chinook salmon at South Santiam Hatchery in 1970.

[c]mg endotoxin per kg of mouse.

Effect of Temperature on the Antibody Response of Juvenile Coho Salmon to Aeromonas salmonicida Endotoxin

In this geographic area, hatchery water temperatures are often low during the spring. To be successful, an immunization program with young juvenile coho salmon must be operated during this season. Therefore, experiments were designed and carried out at 6.7, 12.2 and 17.8°C to determine the immunological competence of juvenile coho salmon at these varied temperatures. The antibody response was followed in fish of two mean weights, 6.5 and 22.6 g.

In these experiments, each animal was injected with 1,000 µg of A. salmoncida endotoxin in an aluminum hydroxide carrier. Antibody response was followed by analysis of serum taken from five or ten individual fish of each temperature group at weekly intervals. Agglutinating antibody levels were determined using the microtiter method.

Tables 24 and 25 show the percent antibody response of each group at the temperatures studied. Figures 4 through 9 show the antibody responses graphically when the mean titer^{-1} is plotted against time. Frequency distributions of antibody titers for various groups are shown in the Appendix.

At 6.7°C, an antibody response was first observed in both sizes of fish four weeks after the antigen injection, but all animals did not respond at this time (Tables 24 and 25, Figures 4 and 5). The highest antibody titers were reached nine weeks after injection.

Prior to the initial response at 6.7°C, agglutinating antibody levels of fish injected with the antigen had decreased below that of the control fish. This effect was emphasized in fish injected with endotoxin and held at 3.9°C. In these animals, antibody levels normally present in control fish had been neutralized by injected antigen leaving titers^{-1} of <10.

At 12.2°C, a response was detected two weeks after antigen administration (Tables 24 and 25, Figures 6 and 7). The response at the two week period was detected in only 50% of the animals tested. Maximum antibody levels appeared four to five weeks after injection. Highest antibody levels were obtained in the larger 22.6 g fish.

At 17.8°C an antibody response could be detected as early as one week after injection (Tables 24 and 25, Figures 8 and 9). In the

experiment with fish weighing 22.6 g each[10], only two of the ten animals had responded by the end of one week. However, three of five of the smaller fish (6.5 g/fish) had responded by this time. Maximum titers were observed in the smaller fish three weeks after injection.

Antibody responses were demonstrated by both ages of juvenile coho salmon at 6.7, 12.2 and 17.8°C. Aeromonas salmonicida endotoxin was non lethal to juvenile coho salmon held at 3.7 to 17.8°C when the ip dosage was 1,000 µg. This indicates that coho can be immunized in hatchery situations even when low water temperatures exist. Although the immune response of these fish would be slower at colder temperatures, it would occur.

[10] This experiment at 17.8°C was terminated prematurely due to a mechanical failure of temperature control equipment.

Table 24. Percent antibody response[a] of juvenile coho salmon[b] at three temperatures to Aeromonas salmonicida endotoxin[c].

Time After Injection (weeks)	Percent Response at Various Temperatures (°C)		
	6.7	12.2	17.8
0	0	0	0
1	0	0	60
2	0	60	80
3	0	80	100
4	20	100	80
5	60	80	60

[a]Percent of a five fish sample with titer$^{-1} \geq 160$

[b]Mean fish weight = 6.5 g/fish

[c]Each animal injected ip with 1000 μg of A. salmonicida endotoxin

Table 25. Percent antibody response[a] of juvenile coho salmon[b] at three temperatures to Aeromonas salmonicida endotoxin[c].

Time After Injection (weeks)	Percent Response at Various Temperatures (°C)		
	6.7	12.2	17.8
0	0	0	0
1	0	0	20
2	0	40	100
3	0	90	100
4	40	100	
5	100	100	
6	80	80	
7	80	100	
8	50	90	
9	80	100	
11	70	100	
13	100	80	
15	50	40	

[a]Percent of a ten fish sample with titer$^{-1} \geq 320$

[b]Mean fish weight = 22.6 g/fish

[c]Each animal injected ip with 1000 μg of A. salmonicida endotoxin

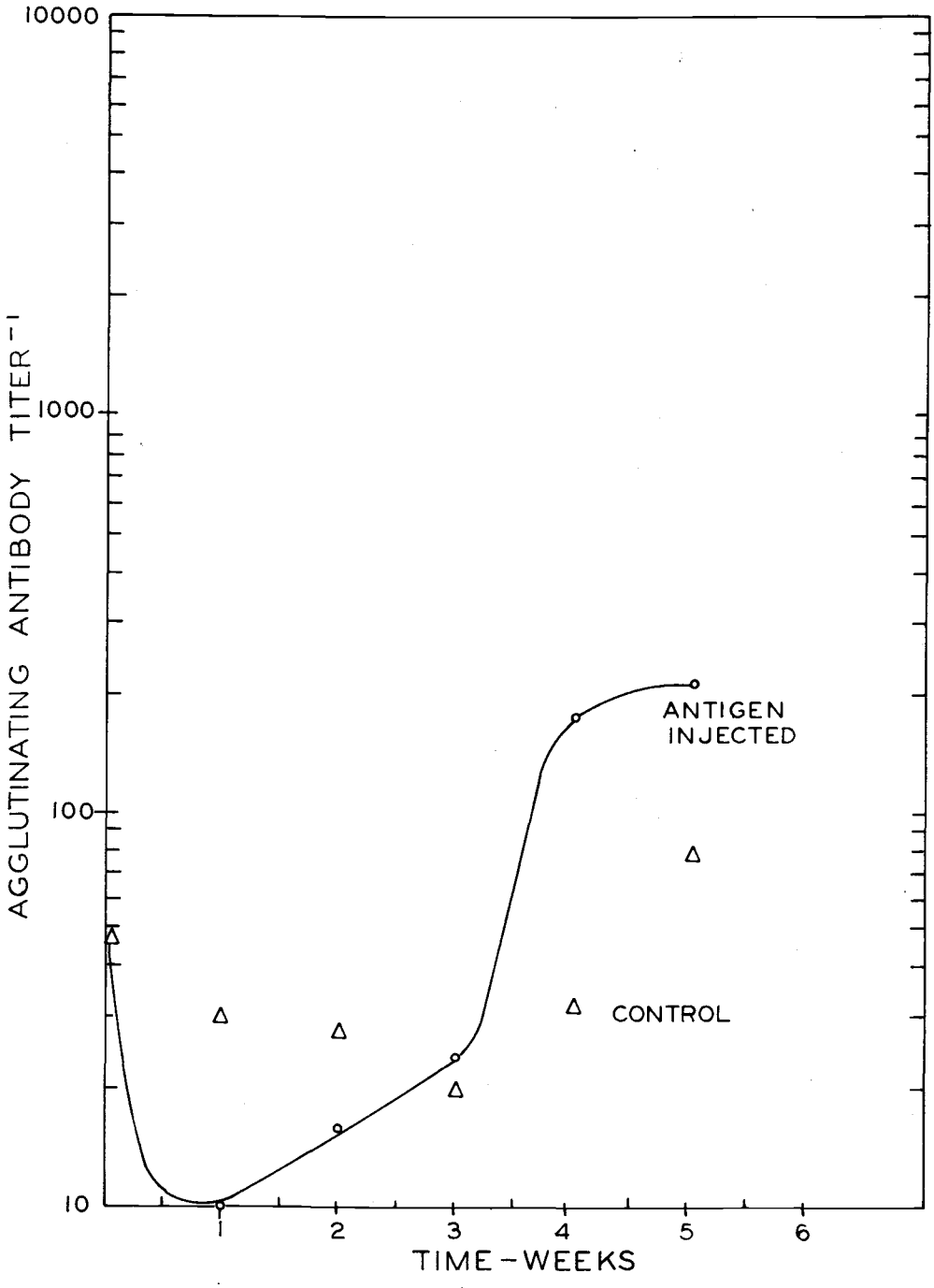

Figure 4. Antibody Response of Juvenile Coho Salmon Held at 6.7°C to a Single IP Injection of Aeromonas salmonicida Endotoxin.
Mean fish weight = 6.5 g
Sample size = 5

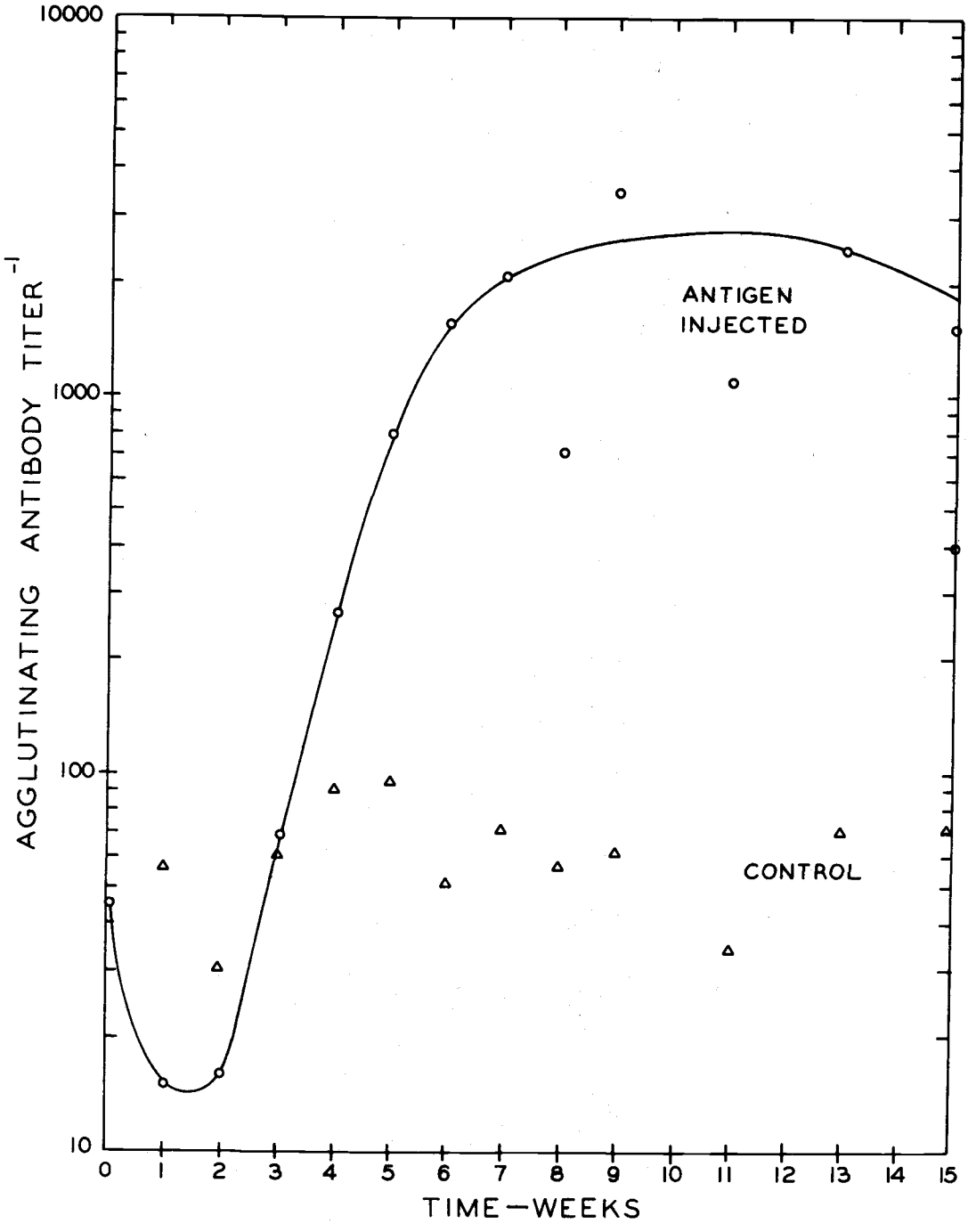

Figure 5. Antibody Response of Juvenile Coho Salmon Held at 6.7°C to a Single IP Injection of Aeromonas salmonicida Endotoxin.
Mean fish weight = 22.6 g
Sample size = 10

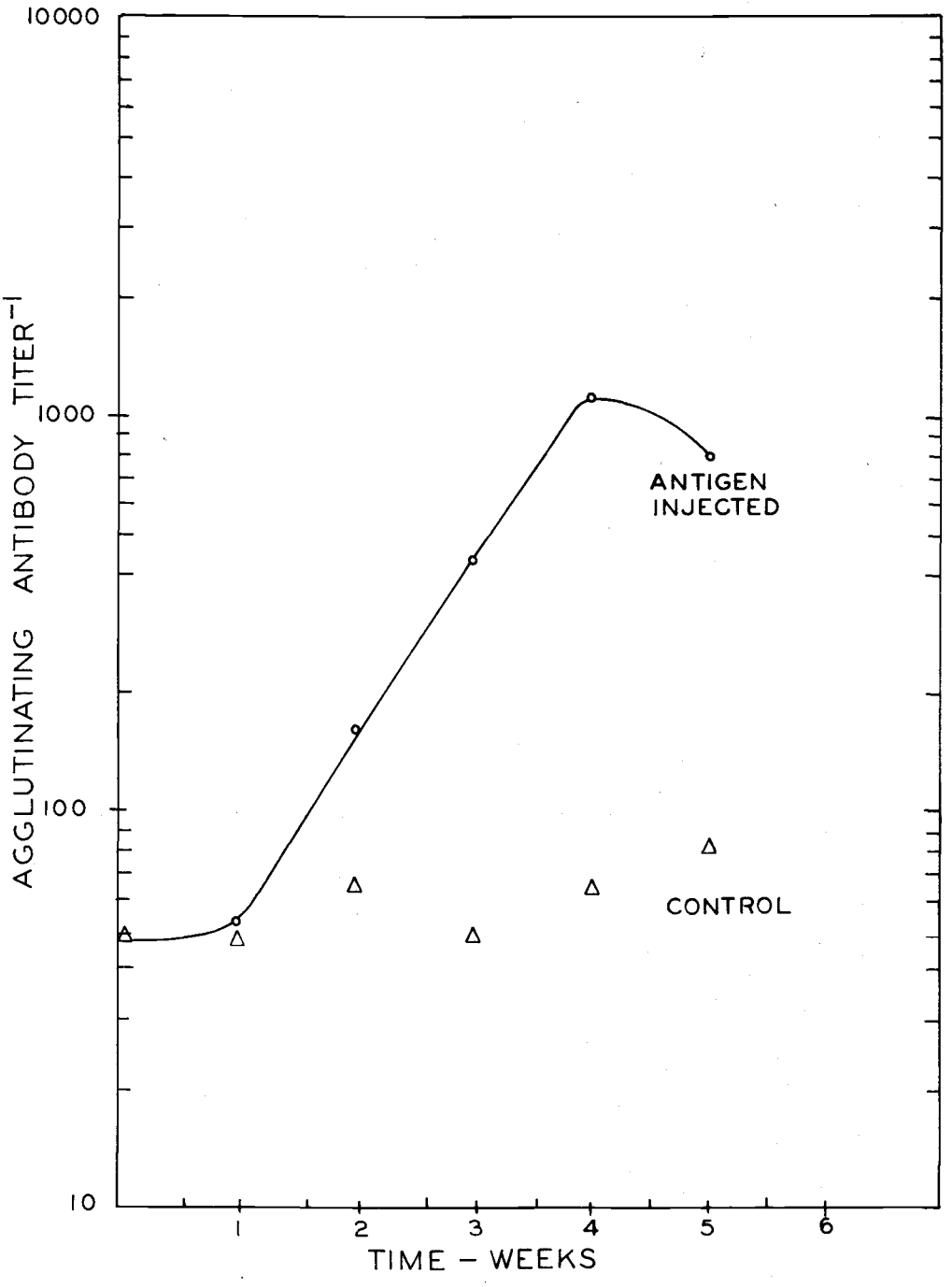

Figure 6. Antibody Response of Juvenile Coho Salmon Held at 12.2°C to a Single IP Injection of Aeromonas salmonicida Endotoxin.
Mean fish weight = 6.5 g
Sample size = 5

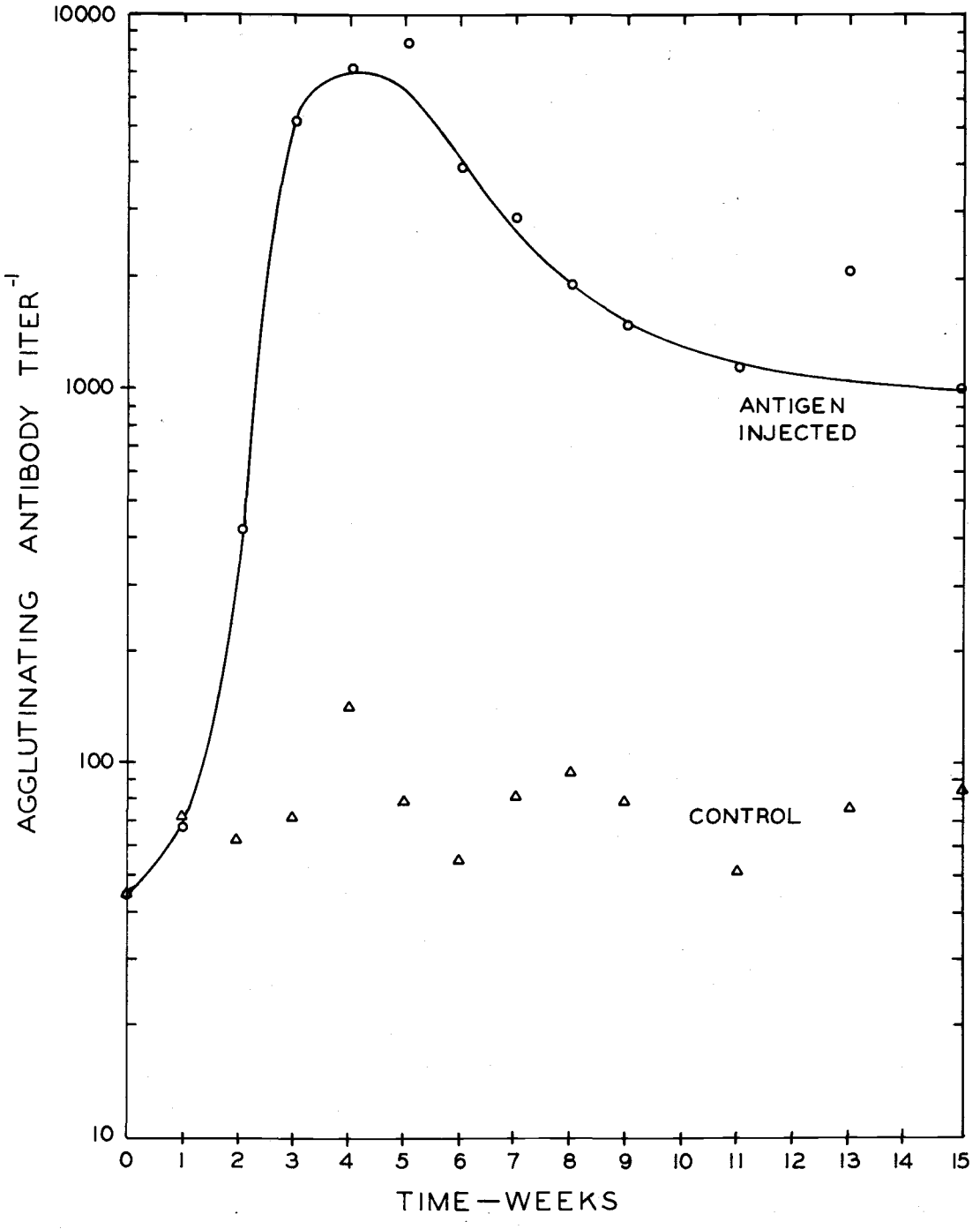

Figure 7. Antibody Response of Juvenile Coho Salmon Held at 12.2°C to a Single IP Injection of Aeromonas salmonicida Endotoxin.
Mean fish weight = 22.6 g
Sample size = 10

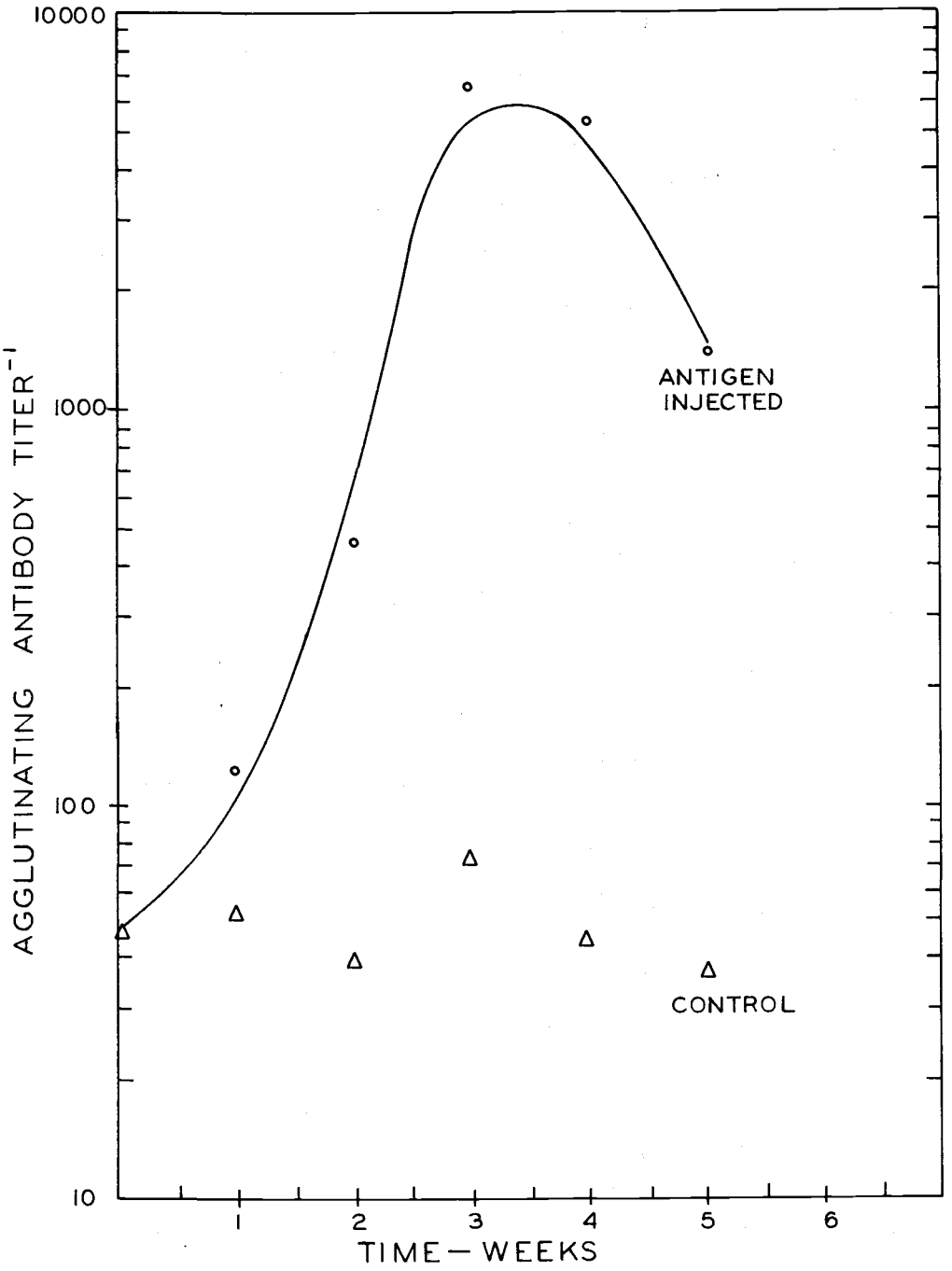

Figure 8. Antibody Response of Juvenile Coho Salmon Held at 17.8°C to a Single IP Injection of Aeromonas salmonicida Endotoxin.
Mean fish weight = 6.5 g
Sample size = 5

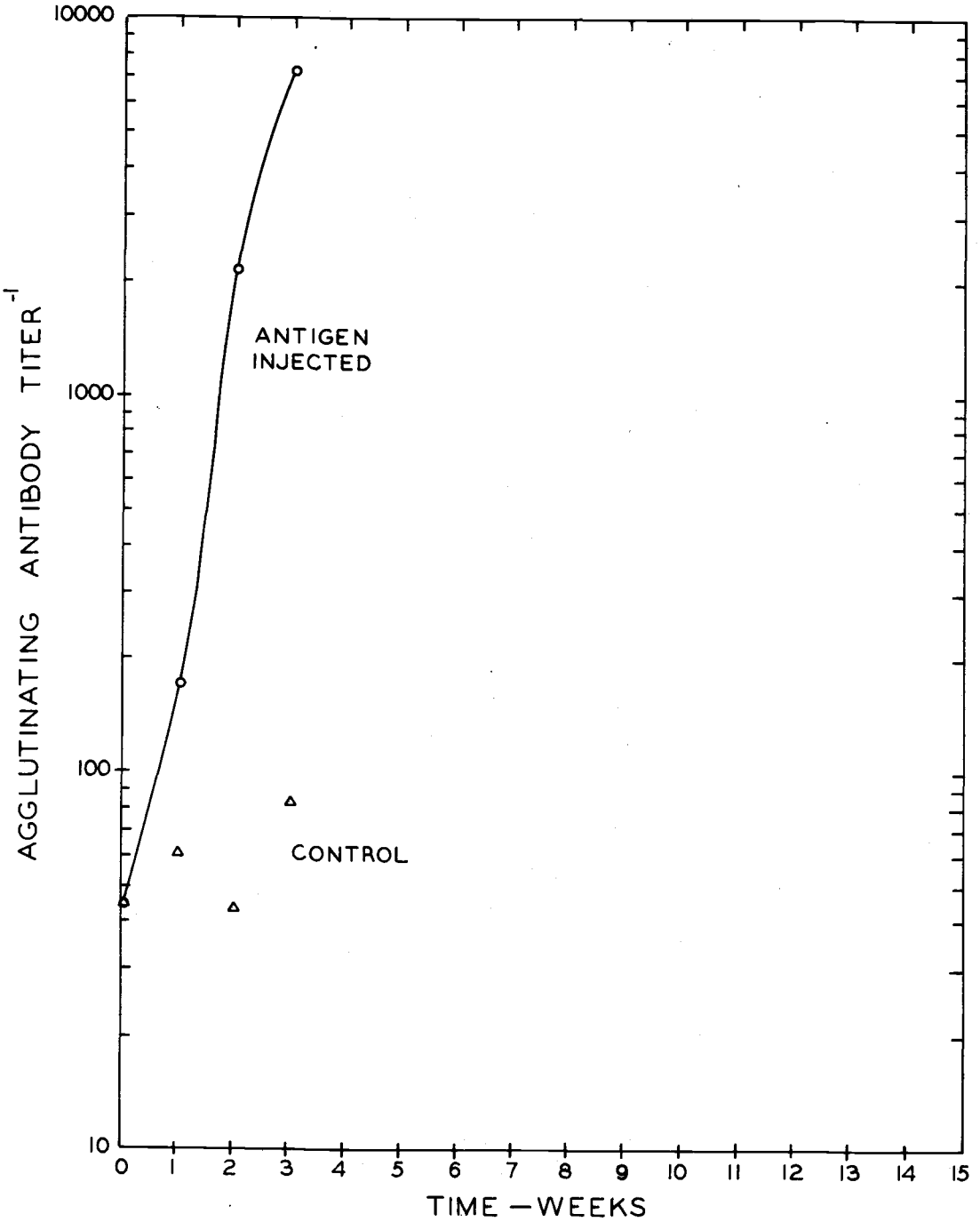

Figure 9. Antibody Response of Juvenile Coho Salmon Held at 17.8°C to a Single IP Injection of Aeromonas salmonicida Endotoxin.
Mean fish weight = 22.6 g
Sample size 10

DISCUSSION

Antigen Production

The yield of cells produced in the Fermacell Fermentor varied from 4.8 to 14.8 g of cells/liter of medium. The maximum yield was obtained by addition of dextrose after medium carbohydrates had been depleted. This condition was indicated by a rise in pH resulting from amino acid metabolism.

Serological Procedures

Results obtained with the microtiter procedure (Table 7) indicated high reliability of this system in monitoring agglutinating antibodies specific for A. salmonicida. The rapid analysis and small sample required allowed the use of this method to monitor serum from individual fish as small as 1.2 g each. The successful monitoring of individual samples of fish serum facilitated experimental design that more completely offered answers to the questions under investigation.

Data obtained from analysis of individual serum samples would allow a worker to project the percent potential immunological competence of large fish populations. If previous data could show response by nearly all of a population, this information would be invaluable in confidently designing and performing immunization experiments of a parenteral or oral nature.

The gel precipitin procedure has been used previously to detect teleost antibodies to soluble A. salmonicida antigens (Anderson and Klontz, 1970; Holway and Klontz, 1971). This procedure is not as sensitive as the agglutination test in a detection of antibodies (Tables 20, 21 and 22). These endotoxin injection experiments involving fish demonstrated the greater sensitivity of the agglutination test.

Antibody Response of Juvenile Coho Salmon to a Single Intraperitoneal Injection of Aeromonas salmonicida Cells Emulsified in Freund's (Complete) Adjuvant

The antibody response observed in coho salmon following injection of a cellular A. salmonicida antigen in FCA was detected later than a response obtained by injection of a soluble antigen (A. salmonicida endotoxin). With an FCA-A. salmonicida cell injection, the first elevated antibody titers were detected four weeks postinjection in animals held at 12.2°C. In contrast, the first elevated antibody levels were observed only two weeks after injection with endotoxin at this temperature.

This later response following an antigen-FCA injection has also been observed by Post, 1963. One possible explanation for this phenomenon is the slow release of antigen when emulsified in adjuvant prior to injection. This antigen neutralizes serum antibody as has been demonstrated at 6.7°C and 3.9°C in the temperature studies of the juvenile coho salmon immune response. On the other hand, the

soluble antigen is neutralized and removed more rapidly from the fish circulatory system. Antibodies then appear rapidly in response to this antigenic stimulation and are not neutralized by pre-existing antigen in the system.

The high antibody titer obtained with injection of A. salmonicida cells emulsified in FCA persists in fish for at least a year after injection. In comparison, titers obtained by injection of endotoxin with only an aluminum hydroxide carrier begin to decrease only five to six weeks after injection. The higher titers and longer duration of antibody response using FCA result from its ability to persist in the animals for long periods of time. This indicates that the antigen is released slowly over an extended period.

Krantz et al. (1963) reported the persistence of antibody titers from 640 to 10,240 in brown trout two years after administration of vaccine emulsified in an oil adjuvant. These workers demonstrated that administration of vaccine without adjuvant produced an antibody response, but antibody titers returned to the pre-immunization level three months after reaching peak titer.

Although protective immunity was demonstrated at the four and six week sampling periods, the ability of an im challenge to detect the immune state is questionable. At the eight and twelve week sampling periods, elevated LD_{50} values were not detected in animals with high agglutinating antibody titer when an im challenge was employed.

The group of juvenile coho salmon injected with cells plus FCA behaved as a very homogeneous population with an overall immune response of 96.6%. Gee and Smith (1941) and Hildemann (1962) reported on the homogeneity of immune response of carp and goldfish, respectively. This type of population response is critical if immunization programs are to be successful.

Oral Immunization Experiment

Although a challenge did not exist to test the oral immunization program, the fish used were shown to be immunologically competent and the sonicated antigen capable of eliciting an immune response in these animals when administered ip. Oral immunization of fish against furunculosis has been successful on only three occasions (Duff, 1942; Overholser, 1968; Klontz and Anderson, 1970). The important ingredients in each case were prolonged feeding of vaccine and a mild natural challenge just capable of killing the majority of control animals. The absence of a suitable challenge has voided this type of experiment frequently. This clearly indicates the need for an efficient challenge procedure that could be utilized in a laboratory at any time of the year to clearly assess the protective value of oral immunization experiments. Before more oral immunization programs are initiated, a concerted effort should be made to perfect a laboratory challenge against furunculosis. Without such a challenge, the progress in

development of oral vaccination procedures against this disease will be minimized.

Investigation of Infection Methods Using a Virulent Isolate of Aeromonas salmonicida

The virulence of A. salmonicida cultures used by investigators to challenge vaccinated fish varies considerably. Snieszko and Friddle (1949) and Krantz et al. (1963) used avirulent cultures (LD_{50} = ~ 10^8 cells) to challenge orally and parenterally immunized fish. If the A. salmonicida South Santiam infected coho kidney tissue was used in a challenge as it was in this work, the LD_{50} was reduced to about a single cell.

The fact that infection of poiklothermic animals rests in a subtle balance is demonstrated by data which positively correlates the presence of certain environmental conditions such as temperature with infection. Overholser (1968) demonstrated the dependence of a furunculosis epizootic on a rise in water temperature to 17.5°C or more. Data in this thesis shows that the LD_{50} rises from 1 to 75 cells when the temperature changes from 12.2°C to 17.8°C.

The failure of oral injection and contact methods of injection indicates that severe conditions of stress (crowding, high temperature, poor water, etc.) must exist before these routes of infection transfer would create an epizootic.

Effect of Various Dosage Levels of *Aeromonas salmonicida* Endotoxin on Juvenile Coho Salmon

Endotoxin of *A. salmonicida* provides an excellent antigenic system for studying the immune response of fish. This antigen was injected into fish and elicited a good immune response without using FCA. This endotoxin was administered quantitatively and elicited a strong antibody response which can be monitored *in vitro* by means of agglutination and precipitin reactions. The high degree of antigenicity of *A. salmonicida* endotoxin for fish is indicated by the positive immune response obtained after injection of only 1 μg.

Aeromonas salmonicida endotoxin is non toxic to juvenile coho salmon under the variety of conditions tested. Endotoxin levels from 1 to 5,000 μg administered in two ip injections caused no deaths. Injection of 1,000 μg of *A. salmonicida* endotoxin at temperatures from 3.7 to 17.8°C caused no mortalities. Free endotoxin found in *A. salmonicida* culture supernatants was also non toxic to these animals. This indicates that endotoxins as represented by this *A. salmonicida* type are non toxic to juvenile coho salmon in the tolerable temperature range of these animals.

Effect of Temperature on the Antibody Response of Juvenile Coho Salmon to *Aeromonas salmonicida* Endotoxin

Juvenile coho salmon synthesized anti-*A. salmonicida* antibodies at 6.7°C, 12.2°C and 17.8°C. First detection of elevated antibody levels was four, two and one week after injection, respectively, for the three temperatures above. This more rapid antibody production at higher temperatures is characteristic of poiklothermic animals.

The rate of antibody synthesis and time of detection after antigen administration to coho salmon are comparable to values reported for eels, trout and goldfish (Muroga and Egusa, 1969; Chiller *et al.*, 1969; Trump and Hildemann, 1970). Muroga and Eguso (1969) demonstrated antibody response of eels to *Vibrio angullarum* in two weeks at 19, 23 and 27°C, and in four weeks at 15°C. Chiller *et al.* (1969) detected an increase in specific antibody forming cells only six days after injection of sheep red blood cells into rainbow trout held between 12 and 17°C. Trump and Hildemann (1970) detected anti BSA antibodies in goldfish in 7 days, 10 days and 17 days at 30, 25 and 20°C, respectively.

At 3.9 and 6.7°C, a marked decrease in antibody level was observed for the first two weeks after injection. This is a reflection on the efficiency of an intraperitoneal injection which circulates throughout the animal and neutralizes serum antibody.

In all but the smallest fish tested, control animals possessed specific antibody titers^{-1} ranging from 10 to 160. Similar observations have been made previously (Bisset, 1948; Frost, 1968; Krantz and Heist, 1970). Bisset (1948) demonstrated agglutinin titers in the sera of perch against bacteria isolated from the peritoneal cavities and opercular cavities of these animals. Frost (1968), Krantz and Heist (1963) detected anti A. salmonicida antibodies in non-immunized trout and salmon.

Several possible explanations for the presence of antibody levels in these control animals could be made. Krantz and Heist (1963) suggested that contact to A. salmonicida in the environment causes the low level of antibodies. Another factor is that the anti A. salmonicida antibodies produced by coho salmon are extremely efficient in agglutinating bacteria. Only 0.04 μg antibody per ml serum is necessary to detect the presence of antibody with these bacteria (Cisar, 1972).

General

The experiments in this thesis have documented some of the immunological capabilities of juvenile coho salmon and provided a confidence factor for future immunization programs. This work showed that the immune state can be obtained by these fish at a variety of temperatures from 6.7 to 17.8°C and in animals as small as 1.2 g. Nearly 100% of the fish tested with an FCA associated vaccine were

immunologically competent. This data demonstrates that hatchery stocks of juvenile coho salmon even of a very young age can be effectively immunized at hatcheries even if low water temperatures prevail. With this assurance that these animals are capable of being immunized under hatchery conditions, investigation should be focused on other problems such as vaccine preparation and application methods.

With regard to oral immunization, there is another area which requires investigation. The type of immune response produced by the administration of an oral vaccine should be characterized. Recent research on secretory antibody and the presence of specific antibody in the mucus of fish suggests the possibility of antibody production of this type in orally immunized fish. Therefore, techniques should be developed to enable workers to monitor the immune response to oral vaccination by analysis of mucus as well as humoral antibodies.

SUMMARY AND CONCLUSIONS

1. The microtiter procedure was adapted for analysis of individual serum samples from juvenile coho salmon of all sizes.
2. High antibody titers and protective immunity were detected four weeks after juvenile coho salmon were injected ip with A. salmonicida cells emulsified in Freund's (complete) adjuvant.
3. These elevated antibody levels were maintained for at least one year after this single injection.
4. An immune response was observed in 96.7 percent of the animals injected with this antigen preparation. This indicates that juvenile coho salmon behave as a homogeneous population with respect to immunological competence to a single intraperitoneal injection of A. salmonicida cellular antigens emulsified in Freund's (complete) adjuvant.
5. Antibody production by animals as small as 1.2 g in response to the above antigen preparation showed that even these very small fish are immunologically competent.
6. Aeromonas salmonicida endotoxin was extracted from Fermacell produced cells using the Boivin procedure.
7. Aeromonas salmonicida endotoxin was non toxic to juvenile coho salmon when 1 to 5,000 μg was administered ip in two doses: when 1,000 μg was injected ip and the fish held at temperatures

from 3.7 to 17.8°C or when free endotoxin was administered im or ip.

8. Aeromonas salmonicida endotoxin was very antigenic to juvenile coho salmon when doses ranging from 1 to 5,000 μg were administered ip with and without an aluminum hydroxide carrier. An antibody response was observed at all levels administered.

9. This endotoxin had a lethal effect on mice when administered by the ip route. LD_{50} values for several preparations ranged from 232 to 536 μg in 18 to 25 g mice.

10. Antibody response to ip administered endotoxin was detected in juvenile coho salmon held at 6.7°C, 12.2°C and 17.8°C. At these temperatures, the first elevated antibody levels were observed four, two and one week after injection, respectively. The response was slower at the lowest temperature, but did occur. Response occurred most rapidly at 17.8°C. This showed that these animals are capable of an immune response over a wide range of temperatures as often exist in hatchery water supplies.

11. Variability of virulence was demonstrated in different cultures of A. salmonicida. LD_{50} values for juvenile coho salmon ranged from 1 to 10^8 A. salmonicida cells.

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APPENDIX

Table A1. Frequency distribution of antibody titers^{-1} of control fish[a] at various time and temperatures after injection with $Al(OH)_3$.

	Frequency of Antibody Titers at Various Times after Injection																	
	6.7°C						12.2°C						17.8°C					
	0	1	2	3	4	5	0	1	2	3	4	5	0	1	2	3	4	5
Titer^{-1}	(weeks)						(weeks)						(weeks)					
<10																		
10		1		2														
20		1	3	2	2									1			1	1
40	4	3	2	1	3		4	4	2	4	2		4	2	5	1	3	4
80	1					5	1	1	3	1	3	5	1	2		4	1	
160																		

[a] Fish weight = 6.5 g/fish

Table A2. Frequency distribution of antibody titers^{-1} at various times after injection of *Aeromonas salmonicida* endotoxin + $Al(OH)_3$ in fish[a] held at 6.7°C.

Time After Injection (weeks)	Frequency of Various Titers^{-1}												
	<10	10	20	40	80	160	320	640	1280	2560	5120	10240	20480
0				4	1								
1	1	2	1										
2		2	3										
3		2	1	2									
4				2	2			1					
5				1	1	2		1					

[a] Fish weight = 6.5 g/fish

Table A3. Frequency distribution of antibody titers^{-1} at various times after injection of Aeromonas salmonicida endotoxin + $Al(OH)_3$ in fish[a] held at 12.2°C.

Time After Injection (weeks)	Frequency of Various Titers^{-1}												
	<10	10	20	40	80	160	320	640	1280	2560	5120	10240	20480
0				4	1								
1			1	2	2								
2					2	2	1						
3					1	1	2		1				
4					1	2				2			
5				1	1			2		1			

[a] Fish weight = 6.5 g/fish

Table A4. Frequency distribution of antibody titers^{-1} at various times after injection of Aeromonas salmonicida endotoxin + $Al(OH)_3$ in fish[a] held at 17.8°.

Time After Injection (weeks)	Frequency of Various Titers^{-1}												
	<10	10	20	40	80	160	320	640	1280	2560	5120	10240	20480
0				4	1								
1				1	1	3							
2					1		3		1				
3									1		2		1
4					1	1		1			1		
5					2	1			1		1		

[a] Fish weight = 6.5 g/fish

Table A5. Frequency distribution of antibody titers^{-1} of control fish[a] at various times and temperatures[b] after injection with $Al(OH)_3$.

Time After Injection (weeks)	Frequency of Antibody Titers^{-1}						
	<10	10	20	40	80	160	320
0		1	2	4	3		
1			2	9	19		
2		3	3	16	8		
3		1		6	22	1	
4				1	9	10	
5		1		9	14	6	
6			2	13	13	2	
7		1	1	6	19	3	
8		1	2	5	16	6	
9				7	23		
11		3	9	5	13		
13		2	7	17	4		
15			2	7	18	3	

[a]Fish weight = 22.6 g/fish

[b]Control fish held at 6.7, 12.2 and 17.8°C

Table A6. Frequency distribution of antibody titers^{-1} at various times after injection of Aeromonas salmonicida endotoxin + $Al(OH)_3$ in fish[a] held at 6.7°C.

Time After Injection (weeks)	Frequency of Various Titers^{-1}												
	<10	10	20	40	80	160	320	640	1280	2560	5120	10240	20480
0		1	2	4	3								
1		5	5										
2		6	3	1									
3			1	2	6	1							
4					2	4	2	2					
5							4	3	2	1			
6						2	2	1	4			1	
7						2	2	2		2	1	1	
8					1	4	2	1		2			
9						1	2		1	5			1
11						3	3		2	1	1		
13								2	3	2	3		
15				2	2	1	1	1		1	2		

[a] Fish weight = 22.6 g per fish, 10 fish/sample

Table A7. Frequency distribution of antibody titers^{-1} at various times after injection of Aeromonas salmonicida endotoxin + $Al(OH)_3$ in fish[a] held at 12.2°C.

Time After Injection (weeks)	Frequency of Various Titers^{-1}												
	<10	10	20	40	80	160	320	640	1280	2560	5120	10240	20480
0		1	2	4	3								
1				3	7								
2						6		3	1				
3						1	4		1	1	1		2
4							3		1	1	1	2	2
5							2	1	2			2	3
6					1	1	3		2		1	1	1
7							4	3		2			1
8						1	4	2	1		1	1	
9							4	2	2		2		
11							5	2	1	2			
13					1	1	1	1	2	1	3		
15					1	5	1		1	1	1		

[a]Fish weight = 22.6 g per fish, 10 fish/sample

Table A8. Frequency distribution of antibody titers^{-1} at various times after injection of Aeromonas salmonicida endotoxin + $Al(OH)_3$ in fish[a] held at 17.8°C.

Time After Injection (weeks)	Frequency of Various Titers^{-1}												
	<10	10	20	40	80	160	320	640	1280	2560	5120	10240	20480
0		1	2	4	3								
1				1	1	6	2						
2							1	3	2	2	2		
3									3	1	1	4	1

[a] Fish weight = 22.6 g per fish; 10 fish/sample