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Title: ORAL AND PARENTERAL IMMUNIZATION FOR THE CONTROL OF VIBRIO ANGUILLARUM, THE ETIOLOGICAL AGENT OF VIBRIOSIS IN SALMONID FISH

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Efficacious vaccines were developed for the control of Vibrio anguillarum, the etiological agent of vibriosis in salmonid fish. These bacterins can be administered either orally or parenterally. It was determined that both formalin-killed lyophilized whole cells and wet-packed whole cells of the organism are effective oral immunogens. Intraperitoneal injection of 0.1 ml containing 2×10^8 formalin-killed bacterial cells suspended in saline and mixed with Freund complete adjuvant is capable of providing protection to fish exposed to natural challenge with V. anguillarum. Oral vaccines prepared from organisms isolated from salmonids in the Pacific Northwest offered greater immunity than bacterins made from European strains when fish receiving them were challenged at Lint Slough, Waldport, Oregon.

Several parameters under which the oral vaccine can be effectively used were examined. These investigations revealed that protection is provided to fish vaccinated for 15 days with a ration containing 0.5 mg of the wet whole cell vaccine per gram. Increasing the number of days the vaccine was fed to as many as 45 days did not increase the degree of resistance in immunized fish. Decreased mortality was also not observed in groups of fish fed a diet containing higher concentrations of vaccine. These studies demonstrated that oral immunization of fish can be successfully accomplished at water temperatures ranging from 4 to 21 C.

Although agglutinating antibody could not be detected in the serum of orally immunized animals, there is an indication that bactericidal antibodies are formed. Fish parenterally vaccinated produced agglutinating as well as bactericidal antibodies.

Oral and Parenteral Immunization for the Control of *Vibrio anguillarum*, the Etiological Agent of Vibriosis in Salmonid Fish

by

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INTRODUCTION

Although aquaculture in the marine environment has been attempted in the past, Vibrio anguillarum, the etiological agent of vibriosis, has been responsible for devastating mortality among cultured fish. Since rearing salmonid fish in mariculture facilities has recently received increased attention, the control of fish disease, and especially the control of vibriosis, is tantamount to developing and maintaining a viable mariculture industry.

Fish cultural management techniques designed to decrease environmental stress and thus reduce the incidence of disease, and the use of chemotherapy are two methods which have been employed in controlling infectious diseases of fish. Each of these methods, however, has inherent problems when animals are intensively cultured in marine or estuarine environments. An early effort to overcome the limitations of chemotherapy and management techniques resulted in the development of an effective oral vaccine against V. anguillarum (Fryer et al., 1972; Nelson, 1972). Based on these studies, the present investigations were undertaken to advance further the concept of oral and parenteral immunization against V. anguillarum.

The objectives of this work were both to develop a more economical and efficacious vaccine and to study the parameters under which such a vaccine could be employed effectively.

This research focused on investigating selected vaccine preparations and conditions of their administration. During the course of these studies, animals received vaccines either by the oral route or by parenteral administration. Experiments included a comparison of the efficacy of the original vaccine with bacterins produced by alternate, more economical and efficient, methods. The effectiveness of selected levels of oral vaccine was examined either by altering the amount of immunogen incorporated into the fish's diet or by changing the duration of the vaccination period. The effect of water temperature on oral immunization was also explored. Throughout the investigations an attempt was made to determine the nature of the protective mechanism provided by oral or parenteral immunization.

LITERATURE REVIEW

Characteristics, Pathology and Distribution of Vibrio anguillarum

The first isolation of Vibrio anguillarum was attributed to Canestrini (1893) who cultured the organism from eels suffering from "Red Pest." It was referred to as Bacterium anguillarum. Bergman (1909) also isolated the organism from eels and was credited with naming the bacterium Vibrio anguillarum. After Bergman's isolation, several other workers described fish pathogens with similar characteristics. These included V. piscium (David, 1927) and Achromobacter ichthyodermis (Wells and ZoBell, 1934). It has been proposed that these and numerous other isolates of V. anguillarum be combined as a single species, Vibrio anguillarum, and be included in the eighth edition of Bergey's Manual of Determinative Bacteriology (Hendrie et al., 1971).

Some of the identifying characteristics of this species are that the organisms are Gram negative, slightly curved rods, and are motile by a single polar flagellum. They are anaerogenic fermenters of glucose and certain other sugars; they are oxidase and catalase positive, and they have a sodium chloride requirement for optimal growth. Members of the genus are sensitive to 0/129 (2, 4, diamino-6, 7-di-iso-propyl pteridine) (Shewan et al., 1954) and to novobiocin.

Nybelin (1935) studied a number of V. anguillarum isolates and suggested two types for the species. Type A produced acid but no gas from mannitol and sucrose and produced indole; type B failed to ferment these sugars and did not produce indole. Smith (1961) subsequently proposed a type C to include those organisms which ferment both mannitol and sucrose but do not produce indole.

Pacha and Kiehn (1969) divided 15 different isolates into three serological groups. The organisms belonging to each of these groups were also related based on their physiological characteristics. By examining DNA homology and base composition of the same organisms, these investigators were able to place the different strains into three groups which corresponded to those they previously described (Kiehn and Pacha, 1969).

The most important characteristic of V. anguillarum concerning the present studies is the organism's pathogenicity to fish. The pathology in salmonids is similar to that produced by Aeromonas salmonicida, the causative agent of furunculosis. Because of this similarity, vibriosis has been referred to as salt-water furunculosis (Rucker, 1963). In salmonids, gross pathology is characterized by erythema at the base of the fins and around the mouth, and the vent is usually reddened and inflamed. Other symptoms include red necrotic lesions of abdominal musculature; petechiae may appear on the body surface, and in more advanced stages ulcerative

hemorrhaging and septicemia occur.

Similar symptoms have been reported in eels (Bergman, 1909). However the pathology in juvenile turbot and brill is not as well defined (Anderson and Conroy, 1970). In acute cases of vibriosis in salmonids, gross pathological symptoms are also less apparent.

Cases of vibriosis have been described in a wide variety of species of fish, and the occurrence of the disease has been reported in Asia, the Americas, and Europe. Anderson and Conroy (1970) have reviewed the geographical distribution of species of fish from which vibrio-associated diseases have been described. These authors report that in 1761, Bonaveri described an outbreak of "Red Pest" which is recognized as the first reference in the European literature mentioning a bacterial fish disease. Vibrio anguillarum has since been reported in fish from many different areas throughout Europe. The number and frequency of these reports indicate the potential economic significance of vibriosis among food fish. Some of the descriptions include isolation of the organism from codling (Gadus callarias) in Denmark (Bagge and Bagge, 1956), from plaice (Pleuronectes platessa) in Germany (Wolter, 1960), and, more recently, from rainbow trout (Salmo gairdneri) in Italy (Ghittino et al., 1972). Haastein and Holt (1972) have isolated the organism from several species of fish from the coastal waters of Norway.

Japanese scientists have devoted much time to the problems

of fish pathology in aquaculture and have made numerous references to the occurrence of V. anguillarum. Hoshina (1956) described an epidemic in rainbow trout and later (Hoshina, 1957) characterized the etiological agent as V. anguillarum. Muroga and Egusa (1967) isolated the organism from ayu (Plecoglossus altivelis) which had died in a salt water lake. In subsequent studies these researchers (Muroga and Egusa, 1970) recovered V. anguillarum from the same species of fish being reared in freshwater ponds. Vibriosis has also caused mortality of eels cultured in fresh water (Jo and Muroga, 1972). Egusa (1969) has included other descriptions of the problem in his review.

Although aquaculture in America is a relatively recent enterprise, attempts to rear salmonids in salt water environments have met with heavy mortality caused by V. anguillarum (Rucker et al., 1953; Rucker, 1959; Wood, 1968; Cisar and Fryer, 1969; Evelyn, 1971). The organism is primarily a salt water pathogen of fish, however it has been isolated from rainbow trout reared in a fresh water hatchery (Ross et al., 1968). In addition to the salmonid species, the etiological agent of vibriosis also has been cultured from diseased winter flounder (Pseudopleuronectes americanus) (Levin et al., 1972).

Immunization of Fish

There has been much progress toward the understanding of the immune system in fish. Researchers (Clem and Leslie, 1969; Grey, 1969; Good and Papermaster, 1964) have found that classes of fish are immunologically competent and have previously reviewed the literature. Many other aspects of fish immunology have been described (Anderson, 1974). The review presented here will be limited to studies concerning vaccination of fish with organisms pathogenic to them.

Parenteral Immunization

Antibacterial agglutinins against A. salmonicida, the causative agent of furunculosis, have been detected in carp (Cyprinus carpio) and in rainbow trout which received a heat-killed bacterin (Smith, 1940). The fish, held in 10 C water, received various regimens of intraperitoneal injections. This investigator noted that at low water temperatures antibody formation was slower than when animals were held in warmer water.

Krantz et al. (1964a) vaccinated brook trout (Salvelinus fontinalis) and brown trout (Salmo trutta) by intraperitoneal injection of formalin-killed A. salmonicida. The bacterin was administered in a single injection either mixed with an adjuvant or in a saline suspension. Antibody titers were higher and more persistent in

groups of fish receiving the vaccine in adjuvant. After maximum titers were reached, animals were challenged by injection of viable A. salmonicida cells. It was found that vaccinated fish had a high degree of resistance to the challenge.

Post (1963) vaccinated rainbow trout with a heat-killed bacterin of A. hydrophila (A. liquefaciens), another fish pathogen. Fish were injected both intramuscularly and intraperitoneally. Although the antibody response was low, the vaccinated animals were significantly protected after an intraperitoneal injection of bacteria.

Passive immunization has been shown to be effective in retarding the progress of infection with A. salmonicida (Spence et al., 1965). These workers prepared immune serum with a high titer in rainbow trout and demonstrated that this serum, when passively injected into coho salmon (Oncorhynchus kisutch) was capable of delaying the onset and severity of furunculosis induced by an artificial challenge.

Golden shiners (Notemigonus crysoleucas) were shown to develop protective antibodies to A. liquefaciens when injected repeatedly with a bacterin prepared from that organism (Summerfelt, 1966). Agglutinating antibodies were detected two weeks after an initial injection of the vaccine; however the fish were not protected until after five immunizing doses had been administered at weekly intervals.

Fujihara and Nakatani (1971) have demonstrated that bacterins prepared from Chondrococcus columnaris are capable of stimulating

the production of antibodies in coho salmon and rainbow trout. Their experiments included the injection of heat-killed cells either subcutaneously or intraperitoneally. They also compared the effectiveness of bacterins suspended in saline and those mixed with Freund adjuvant. The serum from fish injected with the adjuvant-vaccine mixture showed more rapid increase, and ultimately higher, antibody titers than those animals vaccinated with the saline suspension of vaccine.

Schachte and Mora (1973) have also investigated the antigenicity of C. columnaris. These workers studied the immune response elicited by this organism in channel catfish (Ictalurus punctatus), a warm water fish. After either a subcutaneous or intramuscular injection of the bacterin, the serum of the vaccinated fish exhibited a high agglutinating antibody titer.

The most pertinent literature concerning the present investigation was that concerning vaccination of fish against V. anguillarum. Nybelin (1935) reported that the European eel was capable of producing specific agglutinating antibodies to V. anguillarum after being injected several times with heat-killed cells. He found, however, that while the eels did produce antibodies at 18 C, they did not when held at 7 C. Japanese investigators (Muroga and Egusa, 1969) have reached similar conclusions. They found that at 11 C no agglutinating antibodies were formed, but at 15 C high titers were observed

seven weeks after injection with the vaccine. They also showed that vaccinated eels not only developed agglutinating antibodies but also protective immunity against experimental infection. Other Japanese workers (Hayashi et al., 1964; Saito et al., 1964) have also demonstrated that pathogenic vibrios are antigenic as well as immunogenic when used as parenteral vaccines.

Antibody production in plaice has been demonstrated after intraperitoneal injection with bacterins prepared from V. anguillarum (Fletcher and Grant, 1969; Fletcher and White, 1973). A vaccine consisting of heat-killed, sonicated, lyophilized V. anguillarum cells was suspended in Freund complete adjuvant. After one year, antibody titers were measured by means of the passive haemagglutination method. Antibody was not only detected in serum but also found in intestinal and cutaneous secretions. It was determined that parenteral vaccination resulted in high and persistent titers in the serum, while only small amounts of antibody were found in the mucous secretions of the intestine and skin.

Oral Immunization

The subject of oral immunization has been recently reviewed (Klontz and Anderson, 1970; Snieszko, 1970). The authors of each review concluded that although it is possible to immunize fish, this technique of disease prophylaxis needs further investigation before

it can be employed on a production scale.

The first successful report of oral immunization against a fish pathogen was made by Duff (1942). Cutthroat trout (Salmo clarkii) were vaccinated with a chloroform-killed bacterin produced from virulent A. salmonicida cells. The fish were held at water temperatures with means ranging between 7 and 9 C and were fed the vaccine for various lengths of time. Animals were challenged artificially by increasing the water temperature to 19 C and adding virulent bacteria to the water. Results showed that by increasing the number of daily dosages of vaccine from 40 to 70, a greater degree of protection was elicited. Mortality in vaccinated fish was 25 percent while 75 percent of the unvaccinated controls died. This investigator was also able to detect increased serum agglutinating antibody titers in orally vaccinated fish.

Since Duff's description of an effective oral vaccine against A. salmonicida, other researchers have experienced varying degrees of success with bacterins prepared against this pathogen. Snieszko and Friddle (1949) compared the efficacy of an oral vaccine with a chemotherapeutic method using sulfamerazine for the control of furunculosis in brook trout. These researchers reported that oral vaccination was not effective in preventing furunculosis in their experimental animals. However, the vaccine was fed for only eight days before the fish were challenged, and instead of exposure by

adding bacteria to the water, the animals were injected intraperitoneally with A. salmonicida.

Krantz et al. (1964b) also concluded that oral vaccination was unsatisfactory for hatchery immunization programs. In their studies, the effectiveness of the oral bacterins (viable cells and chloroform-killed cells) was evaluated only by the appearance of agglutinating antibodies. The orally vaccinated fish were not challenged. Similar results were obtained by Spence et al. (1965). No agglutinating antibodies were demonstrated, and oral vaccination of coho salmon provided no protection when the animals were artificially infected.

Klontz (Klontz and Anderson, 1970) developed an oral bacterin against A. salmonicida which was effective in protecting salmonid fish against furunculosis. The antigen used was an alum precipitate of the water soluble portion of sonicated bacterial cells. In laboratory experiments the bacterin elicited a high degree of protection against A. salmonicida. In subsequent field trials the effectiveness of this preparation as an oral immunogen was equivocal.

A small production scale immunization program was done with the vaccine developed by Klontz (Overholser, 1968). In this study, protective immunity was elicited by the bacterin, and only 0.7 percent of immunized fish died from furunculosis; there was a 37 percent mortality in unvaccinated control animals.

Oral vaccines have also been prepared against other fish

pathogens. One of these is a bacterin against the etiological agent of "Redmouth Disease," an organism which has not been classified but has many characteristics of enteric bacteria. In preliminary studies with oral bacterins of this pathogen, Ross and Klontz (1965) reported a significant increase in resistance in vaccinated animals when compared to nonvaccinated fish. This vaccine was further developed by Anderson and Ross (1972) when they explored the effectiveness of four bacterin preparations. Even though the animals displayed no serum agglutinating antibodies, they found that each vaccine tested was capable of eliciting protective immunity to fish challenged by subcutaneous injection with the disease causing organism.

Fujihara and Nakatani (1971) have reported success in oral immunization of fish against C. columnaris. A preparation of heat-killed C. columnaris cells was fed to fish over an extended period of more than 17 weeks; mortality in vaccinated fish was eight percent compared to 48 percent in control animals.

Schaperclaus (1972) has extensively studied the immunization of carp against A. liquefaciens. Several different antigens prepared from this organism were effective in producing protective immunity in vaccinated fish. Although he was able to detect high agglutinating antibody titers after fish had been parenterally injected with the bacterins, there was no antibody formation above background titers

in orally immunized animals.

Oral vaccination of fish against V. anguillarum has been studied by several Japanese investigators (Endo, 1961 and Hayashi et al., 1964). These studies revealed that fish immunized with formalin-killed preparations of V. anguillarum were able to withstand artificial and natural challenges better than unvaccinated fish.

A lyophilized sonicate of the bacteria has been used as an efficacious vaccine (Nelson, 1972; Fryer et al., 1972). This bacterin was administered at various levels and was capable of reducing mortalities in fish exposed to a natural challenge of vibriosis. It was subsequently found that the Boivin antigen, prepared by trichloroacetic acid extraction of V. anguillarum cells, was also an effective immunogen (Abe, 1972).

The antibody production in plaice after oral vaccination with a lyophilized sonicate of V. anguillarum has been investigated (Fletcher and White, 1973). The fish were fed the bacterin daily for a period of one year. Antibodies were detected by passive haemagglutination, and it was found that very low titers existed in the serum of vaccinated animals. However, when intestinal mucous was tested, a higher antibody titer was demonstrated. The converse of this was found in fish which had been vaccinated by intraperitoneal injection.

MATERIALS AND METHODS

Culture Media

Throughout these studies several media were used for the routine culture of V. anguillarum, and the organisms grew sufficiently on each. Furunculosis Agar and Brain Heart Infusion Agar (BHI) were formulated according to the recommendations of the manufacturers. Tryptic Soy Broth was the media used for most of the mass culturing done. These three formulations were purchased from Difco Laboratories, Detroit, Michigan. However, in later experiments, in an effort to reduce the cost of producing large quantities of bacteria, another preparation with components similar to those of Tryptic Soy Broth was used. This medium consisted of 1 percent tryptone, 0.5 percent yeast extract, 0.25 percent sodium chloride in distilled water.

Because it is difficult to retain viability of V. anguillarum by lyophilization, stock cultures were maintained on Cytophaga Sea Water Agar (Pacha and Ordal, 1967). This medium consists of 0.02 percent beef extract, 0.05 percent tryptone, 0.05 percent yeast extract, 0.02 percent sodium acetate, 2.5 percent Rila salts, and 0.4 percent agar in distilled water. It was dispensed into screwcap tubes and used as agar deeps.

Bacterial Cultures

An extensive collection of V. anguillarum isolates was obtained during these studies. Typical isolates from Lint Slough were collected during annual epizootics. Isolates were also received from various locations including several from mariculture operations in Puget Sound, one from a similar facility in Alaska, and from various other coastal areas of the United States. Also included in this culture collection is the organism isolated by Bagge and Bagge (1956) from Denmark and a culture of V. anguillarum derived from an epizootic of vibriosis in Italy (Table 1). All isolates were maintained in Cytophaga Sea Water Agar deeps at 4 C and transferred annually. Most of the vaccines used for immunization of fish at Lint Slough were prepared from the most recent isolate from that location. A typical isolate of V. anguillarum is depicted in Figure 1.

Table 1. Isolates of Vibrio anguillarum used in these studies.

Culture collection number	Location where isolation was made	Person responsible for isolation
LS272	Lint Slough Waldport, Oregon	J. Rohovec
LS173	Lint Slough Waldport, Oregon	J. Rohovec
MAN173	Clam Bay Manchester, Washington	A. Novotny
LUM172	Lummi Aquaculture Bellingham, Washington	K. Johnson
ITA172	Italy	P. Ghittino
BB156	Denmark	Bagge and Bagge (ATCC 19264)

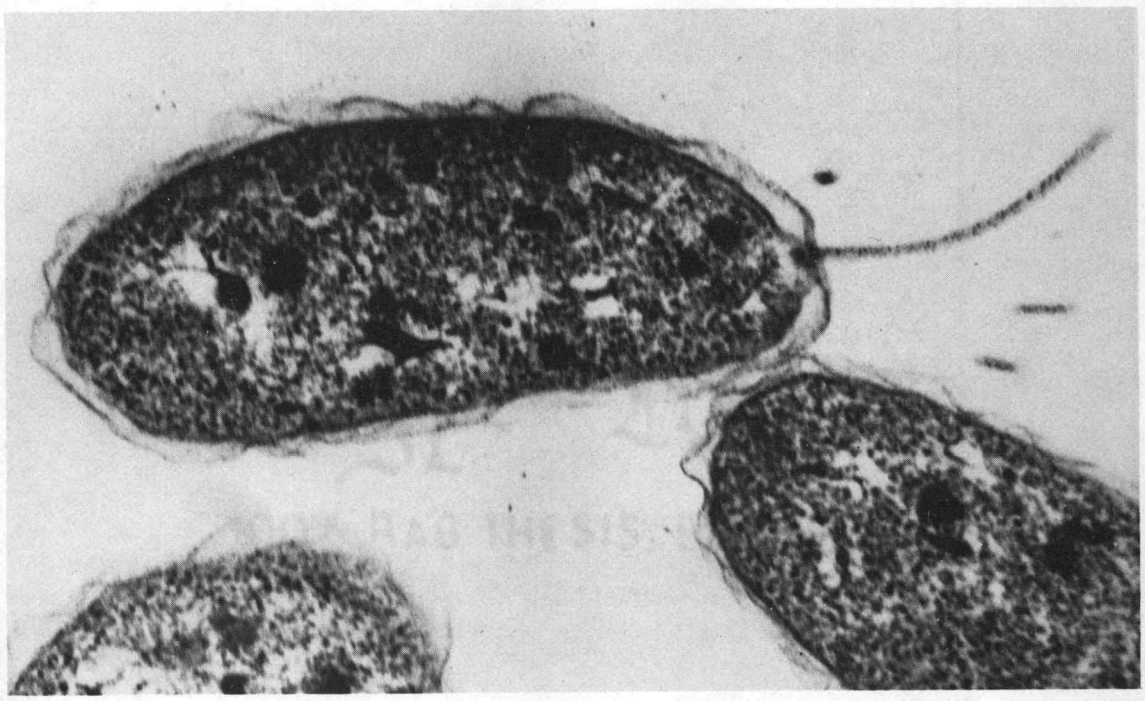

Figure 1. Electron Micrograph of a thin section of Vibrio anguillarum (68, 800 X). Cells were fixed in one percent osmium tetroxide and three percent glutaraldehyde. Pellet fragments were imbedded in agarose and dehydrated in an acetone series to 70 percent, stained in uranyl acetate in 70 percent acetone, dehydrated in acetone, embedded in an araldite-epon mixture, sectioned and post-stained with alkaline lead citrate. A Phillips model 300 was used for microscopy.

Vaccine Preparation

Mass Culture Techniques

A 10 ml broth culture was prepared by inoculation from the desired stock culture and incubated at 28 C for 12 hours. This culture was examined for purity using the Gram reaction and by observation with phase contrast microscopy. Two ml of this bacterial suspension were used to inoculate each of two, one liter quantities of sterile broth which were then incubated 12 hours on a shaker. The cultures were examined for purity as before. The two liters of broth culture were used to inoculate 30 liters of broth which had been sterilized in a Fermacell Fermentor Model CF-50 (New Brunswick Scientific Co., Inc.). This was then aerated aseptically at a rate of 5.7 liters of air per minute and agitated with an impeller at 200 rpm. An incubation period of between 10 and 12 hours at 28 C was allowed to elapse before addition of 500 ml of a sterile 20 percent glucose solution. The 30 liter culture was incubated for an additional 12 hours. Again the purity was examined microscopically and by the rapid slide agglutination method using specific antiserum. Harvesting the bacterial cells from this broth culture was accomplished using a continuous flow Sharples Super Centrifuge Type 1P. Approximately 500 gm of wet packed cells could be obtained from 30 liters of broth using these techniques.

Preparation of Vaccines

Three types of oral vaccines were used in these studies. These included (1) lyophilized sonicate of the bacterial cells, (2) lyophilized whole cells, and (3) wet-packed whole cells. The first step in the preparation of each of these vaccines was to grow V. anguillarum in mass culture. Nelson (1972) described the preparation of the lyophilized sonicate. Two hundred fifty gm of the harvested cells were resuspended in one liter of a saline solution containing 0.3 percent formaldehyde and mixed for 24 hours. A 0.1 ml aliquot of this suspension was placed on an agar medium and incubated for 48 hours. The absence of growth indicated nonviability of the bacterial cells. The killed bacterial suspension was ultrasonically disrupted (Branson W185 Sonifier Cell Disruptor with the W horn, Heat Systems, Inc.) by passing it through a 50 ml continuous flow disruptor vessel at a flow rate of 25 ml per minute. A power setting of eight was used at 100 watts. The sonicated suspension was then lyophilized (VirTis Model 10-145 Lyophilizer, VirTis Research Equipment, Inc.) and stored at room temperature (25 C). The lyophilized whole cell vaccine was prepared using the same methods without sonication. For preparation of the wet-packed whole cell vaccine, cells were killed in the fermentor by adding 100 ml formaldehyde solution to the 30 liter broth culture and allowing it to mix for one hour. Viability

tests were performed and the cells harvested by high speed centrifugation. The wet packed cells were frozen, stored at -26 C and used as the third type of vaccine.

Vaccines were also prepared for parenteral administration. These immunogens were used both for the vaccination of fish and for the production of specific antibodies in rabbits. Because smaller quantities of bacterin were needed for this type of immunization, the V. anguillarum cells were cultured on agar surfaces. This was done by preparing agar slants in eight oz prescription bottles which were inoculated with 1.0 ml of a 12 hour broth culture of the desired organism. After incubation, the bacteria were removed from the agar surface with 0.3 percent formalin-saline solution. The cells remained in this solution for one hour and were then washed three times by centrifugation (0 C, 3,000 g, 10 min) in phosphate buffered saline (PBS) (Williams and Chase, 1968).

General Methods of Vaccination and Challenge

Experimental Animals

Juvenile fall and spring chinook salmon (Oncorhynchus tshawytscha) and juvenile coho salmon (O. kisutch) were used as experimental animals. Spring chinook were obtained from the Willamette River Salmon Hatchery (Fish Commission of Oregon).

All other fish employed in these studies were supplied by the Oregon Wildlife Commission Research Laboratory, Corvallis, Oregon. The size of these experimental animals varied depending on the stock and time of the year in which they were used.

Fresh Water Facilities Used for Immunization

The Oregon State University Fish Disease Laboratory, Corvallis, Oregon offered ideal facilities for conducting the fresh water phase of the experiments. The laboratory is equipped with one meter diameter, circular fiberglass tanks furnished with pathogen-free well water at an ambient temperature of 12 C. There is also the capability of varying the water temperature supplied to experimental aquariums from 4 to 23 C.

Vaccination Procedure

In each experiment, oral vaccines were incorporated into the fish's ration at selected levels per unit of diet. The rations used were Oregon Test Diet (OTD) (Lee et al., 1967), a semi-synthetic diet and Oregon Moist Pellets (OMP) (Hublou, 1963), a commercially available diet. The amount of ration that was to be fed was estimated using feeding charts supplied by the Fish Commission of Oregon (Tables 2 and 3). Because fish are poikilotherms their metabolism is variable and dependent on water temperature. These feeding

Table 2. Oregon moist pellet feeding chart.[a] Estimated amount of food that salmon fingerlings will consume if held at constant water temperatures and fed two times per day, seven days per week.[b] Feeding rate expressed as percentage of body weight.

Water temperature (°F)	Weight of fish (number per pound)							
	305 to 230	230 to 180	180 to 140	140 to 115	115 to 90	90 to 75	75 to 65	65 to 55
39	2.10	1.80	1.70	1.55	1.40	1.25	1.10	1.00
44	3.10	2.80	2.70	2.55	2.40	2.20	2.10	2.00
49	4.80	4.35	4.10	3.80	3.60	3.25	2.95	2.80
54	6.20	5.55	5.15	4.80	4.40	4.10	3.75	3.60
59	7.30	6.70	6.10	5.75	5.35	4.95	4.50	4.35
64	8.30	7.70	7.10	6.75	6.10	5.70	5.25	5.10
69	9.30	8.70	8.10	7.75	6.85	6.45	6.00	5.85

[a] Provided by D. A. Leith, Biologist, Fish Commission of Oregon.

[b] Based on laboratory experiments with coho fingerlings held at constant water temperatures.

Table 3. Oregon Test Diet Feeding Chart.[a] Estimated amount of Oregon Test Diet salmon fingerlings will consume if held at constant water temperatures. Feeding rates are expressed as percentage of body weight.

Water temperature (°F)	Weight of fish (number per pound)			
	300 to 200	200 to 135	135 to 90	90 to 6
53	5.1	4.2	3.4	3.2
54	5.4	4.4	3.6	3.3
55	5.7	4.6	3.8	3.5
56	6.0	4.8	4.0	3.7
57	6.3	5.0	4.2	4.0

[a]Provided by J. H. Wales, Associate Professor of Food Technology, Oregon State University.

charts are based on the temperature of the water and also on the body weight of the animals. The fish are fed a percentage of their body weight depending both on the animals' weight and the temperature at which they are held. Because all diets are not equally consumed, the percent body weight of food fed also depends on the type of ration offered. The total amount of food to be fed is calculated in the following manner:

$$\text{grams of diet fed} = \frac{\text{number of fish}}{\text{number of fish/lb}} \times 454 \text{ gm/lb} \times \frac{\text{percent}}{\text{body weight}}$$

Rations with incorporated vaccine were fed over specified periods of time to fish maintained at the fresh water facility described. Groups of control animals were fed the same diet containing no vaccine. All experiments were done at water temperatures of 12 C unless otherwise specified.

In all cases when parenteral immunization was employed, the fish received an intraperitoneal injection of the vaccine. The vaccine consisted of the preparation previously described. It was diluted to an optical density of 0.85 at 525 nm (approximately 5×10^9 cells/ml) and mixed 1:1 with Freund complete adjuvant (Difco Laboratories, Detroit, Michigan). The fish were anesthetized with a 0.025 percent solution of methyl pentanol and injected with 0.1 ml of the adjuvant-vaccine mixture.

After either oral or parenteral vaccination, the animals were

held in fresh water for some specified length of time. In the case of parenterally immunized fish, this was done to allow time for the protective mechanism to respond and for antibodies to be produced. After oral vaccination, this post-immunization period was employed for this same reason and also to enable fish to clear the antigen which had been fed. Because the mechanism of protection is not fully understood, this precaution was taken to insure that the vaccine would not interfere with the immune system. For example, the vaccine could bind protective antibodies or block macrophages which would otherwise be available to resist infection.

Salt Water Facilities Used for Challenge

After the vaccination period, the experimental animals were challenged at Lint Slough, a salt water rearing impoundment which is located at Waldport, Oregon and is operated by the Oregon Wildlife Commission. This facility offered a unique natural challenge to V. anguillarum with epizootics of vibriosis which have caused mortalities of 90 percent (Cisar and Fryer, 1969). The mortalities due to vibriosis were much higher during the summer months when the water temperature of the slough was above 12 C, indicating that the progress of the disease is temperature dependent. Although very little data were collected, the number of V. anguillarum present in the water ranged from approximately 0.2 to 1.0×10^3 organisms/ml

during the summer months. At Lint Slough, experimental groups of fish were held in one meter diameter, circular fiberglass tanks supplied with water pumped directly from the slough. The duration of the exposure period ranged from 20 to 40 days. During this time a heavy loss occurred between the fourth and tenth days after the fish were introduced to salt water, and when the experiments were terminated increasing mortality had ceased. The availability of tanks at Lint Slough and also at the fresh water facility was often the limiting factor in experimental design.

Examination of Experimental Animals

Each fish that died during the challenge was collected on the day of its death and kept frozen until a necropsy was performed. The dead fish were examined for gross pathological symptoms, dissected aseptically, and bacteriological cultures were then prepared from kidney tissue using one of several types of agar media (BHI, Furunculosis, or Tryptic Soy). After 24 hours incubation at 30 C, the plates were examined for typical colonies of V. anguillarum. Presumptive tests on these cultures included microscopic examination with the Gram reaction, and also morphology and motility using phase contrast microscopy (1000 X). Infection by V. anguillarum was confirmed with rapid slide agglutination tests using the suspected isolates as antigens and V. anguillarum antiserum which had been

prepared in rabbits. Using these tests, a high percentage of the mortality of experimental animals was attributed to V. anguillarum.

Description of Specific Oral Vaccination Experiments

Comparison of Various Vaccine Preparations

The goals of these earlier experiments were two-fold. First, to determine whether the efficacy of immunization could be improved by altering the vaccine, and second to produce a more economical bacterin which would be as effective as the lyophilized sonicate used in the original studies (Nelson, 1972; Fryer et al., 1972). Three preparations of oral bacterins were employed. These were the lyophilized sonicate and lyophilized whole cells; both of these had been formalin-killed. Also included was a lyophilized sonicate which was not treated with formalin; however the sonication period was increased from two to five minutes.

Each of the oral vaccines was incorporated into OTD at a level of two mg of vaccine/gm of diet. Groups of 200 spring chinook salmon (mean weight 3.8 gm) were fed 30 gm of diet/200 fish per day. Groups of unvaccinated control fish received OTD containing no vaccine. These different preparations were fed for 30 days. After the vaccination period, all fish were maintained in fresh water for 15 days before being challenged in salt water.

All groups of fish were simultaneously exposed to the natural challenge of V. anguillarum at Lint Slough and were continually challenged for 30 days. After 20 days exposure, an additional group of control animals was exposed to insure that a sufficient challenge continued to exist.

In a subsequent vaccine comparison, the lyophilized sonicate and lyophilized whole cell bacterins were fed to groups of 200 fall chinook salmon (mean weight 0.3 gm). The efficacy of these two preparations was compared to wet whole cell vaccine which was fed to a similar group of fish. Both lyophilized preparations were incorporated into OTD at a level of two mg of vaccine/gm of ration. Dry weights were determined on ten batches of vaccine and it was demonstrated that the wet packed preparation contained approximately 80 percent water (Table 4). Therefore this bacterin was incorporated at a level of ten mg of vaccine/gm of OTD. Each group of fish was fed one of these diets for 45 days while a control group was fed OTD containing no vaccine. After the vaccination period, the animals were maintained in fresh water for 15 days before being transported to Lint Slough where they were challenged for 40 days.

Determination of the Effectiveness of Selected Oral Vaccination Periods

Fish were orally vaccinated for different lengths of time to determine whether an increased number of vaccine dosages would

Table 4. Dry weight determinations of ten batches of wet-packed, whole cell Vibrio anguillarum vaccine.[a]

Batch number	Wet weight	Dry weight[b]	Mean percent moisture	Mean percent dry weight
	0.18936	0.03496		
1	0.29216	0.05436	81.4	18.6
	0.26472	0.04988		
	0.11161	0.02556		
2	0.09531	0.02145	77.6	22.4
	0.06755	0.01465		
	0.10076	0.02236		
3	0.04535	0.00920	78.7	21.3
	0.07305	0.01576		
	0.13900	0.02637		
4	0.05637	0.01125	80.7	19.3
	0.06854	0.01297		
	0.14934	0.02811		
5	0.13265	0.02998	79.7	20.3
	0.05566	0.01081		
	0.09144	0.01845		
6	0.10237	0.02138	79.6	20.4
	0.06384	0.01274		
	0.07967	0.01550		
7	0.17353	0.02904	81.5	18.5
	0.11602	0.02241		
	0.08602	0.01597		
8	0.13705	0.02411	81.7	18.3
	0.06729	0.01252		
	0.10469	0.02255		
9	0.10262	0.02061	79.6	20.4
	0.07513	0.01472		
	0.10905	0.02217		
10	0.12758	0.02578	80.2	19.8
	0.08294	0.01577		

[a] Determinations done by C. T. Pinney, Jr.

[b] After drying to constant weight at 105 C in a desiccator.

result in increased protection when the animals were challenged. The lyophilized whole cell vaccine was added to OMP at a level of two mg of vaccine/gm of ration and fed to groups of 200 fall chinook salmon (mean weight 0.3 gm). One group of animals was administered this diet for 15 days, one for 30 days, and one for 45 days. The vaccination periods were begun so that each experimental group received its last vaccine dosage on the same day. All immunized fish and an unvaccinated control group were then maintained on OMP for 15 days before being simultaneously challenged for 40 days at Lint Slough.

Another experiment was done with a similar design. In this study, lots of 100 coho salmon (mean weight 15 gm) were fed OTD containing two mg of wet whole cell vaccine/gm of ration. The vaccination periods studied were 1, 2, 5, 10, and 15 days. After immunization, all groups were maintained one week in fresh water before being challenged at Lint Slough for 20 days.

Determination of the Effectiveness of Selected Oral Vaccine Concentrations

Further studies were done to determine whether increasing vaccine dosage would provide greater protection to experimental animals. Groups of 200 fall chinook salmon (mean weight 6.5 gm) were fed wet whole cell vaccine at levels of 2, 5, or 10 mg/gm of

OTD for 30 days. The fish remained in fresh water for 15 days and then were challenged at Lint Slough for 30 days.

In a subsequent experiment exploring vaccine concentrations, lower levels of vaccine were used. In this study, lots of 100 coho salmon (mean weight 12.6 gm) were fed 0.5, 1.0, 2.0, and 5 mg of the wet whole cell vaccine/gm of OTD. The vaccine was fed for only 15 days, followed by five days in fresh water on a diet (OMP) without vaccine. The fish were then exposed to V. anguillarum for 20 days at the salt water rearing facility.

Immunization at Selected Water Temperatures

Variations in water temperatures are encountered among hatchery locations and with the seasons of the year. It has been reported that agglutinating antibody production in poikilothermal animals is slower at low temperatures (Cushing, 1942; Avtalion, 1969; Paterson and Fryer, in press). Because all of the immunization experiments had been conducted in water temperatures of 12 C, it was desirable to determine if oral vaccination could be accomplished at other temperatures. The temperature control equipment at the Oregon State University Fish Disease Laboratory consists of Bristol proportional controller-recorders and mixing valve assemblies which are capable of mixing the ambient water (12 C) with heated or

chilled water. Desired water temperatures in aquariums are maintained automatically to ±0.3 C. Lots of coho salmon (mean weight 6.5 gm) were tempered from 12 C to each of seven temperatures, to include: 3.9, 6.7, 9.5, 12.2, 15.0, 17.8, and 20.6 C.[1] For 15 days these animals were fed OMP containing two mg of wet whole cell vaccine/gm of ration. The ration was fed ad libitum, and because metabolism varies with temperature, the different groups of fish received varying amounts of food (Table 5).

Table 5. Amount of diet and vaccine consumed in 15 days by groups of 100 coho salmon (mean weight 6.5 gm) held at selected water temperatures.

Temperature (°C) at which fish were held	Total grams of diet consumed	Total milligrams of vaccine consumed
3.9	157	314
6.7	264	528
9.5	357	714
12.2	366	732
15.0	490	980
17.8	450	900
20.6	423	846

This feeding schedule simulates actual hatchery regimens and was derived from the feeding charts previously described. After the

[1]The Oregon State University Fish Disease Laboratory is designed to record in degrees Fahrenheit; these temperatures correspond to 39, 44, 49, 54, 59, 64 and 69 F.

fish were administered the vaccine, they were tempered back to 12 C. Tempering of fish took place during a one week period at temperature increments of 3 C every two days. After all groups had been returned to 12 C, they were maintained at this temperature for one week before being exposed to V. anguillarum at Lint Slough. The vaccinated fish were compared to a similar unimmunized control group which had been held at 12 C throughout the fresh water phase of the experiment.

The experiment was repeated with three modifications of the design described above. First, the fish used in this experiment were smaller (mean weight 3.3 gm), therefore these animals consumed less diet than those used in the previous study (Table 6). Secondly, replicate groups of animals were employed. The third modification of the experimental design was that after the diet containing vaccine was fed for 15 days, each group of fish was held at its respective temperature for one week before being tempered back to 12 C. The fish were challenged naturally at Lint Slough for 20 days.

Table 6. Amount of diet and vaccine consumed in 15 days by groups of 100 coho salmon (mean weight 3.3 gm) held at selected water temperatures.

Temperature (°C) at which fish were held	Total grams of diet consumed	Total milligrams of vaccine consumed
3.9	73	146
6.7	118	236
9.5	159	318
12.2	141	282
15.0	320	640
17.8	297	594
20.6	345	690

Determination of the Effectiveness of Vaccines Prepared from Selected V. anguillarum Isolates

Because V. anguillarum is widely distributed, abbreviated in vivo studies were undertaken to determine if differences in protective antigens exist in isolates from various geographical locations. Wet whole cell vaccines were prepared from several isolates, one from Lint Slough, two from different locations on Puget Sound (Lummi and Manchester), and two from Europe (Denmark and Italy). Each of these bacterins was incorporated into OTD at a level of two mg/gm of ration and fed to fall chinook salmon (mean weight 15 gm) for 30 days. The period after vaccination and before exposure was 15 days. All groups were challenged naturally at Lint Slough.

A similar in vivo test was done to determine whether antigenic variation occurred in organisms which were isolated in different years. In addition to this objective, the experiment was also designed to determine if vaccines remain effective after prolonged storage. Oral bacterins were prepared from two Lint Slough isolates (LS272 and LS173) which were derived from epizootics occurring in consecutive years. From these two isolates, three wet whole cell vaccines were prepared: (1) vaccine prepared in 1972 from LS272 and stored at -26 C for approximately one year, (2) vaccine prepared in 1973 from LS272 but made just prior to the time the experiment was conducted, and (3) vaccine prepared from LS173, also made immediately prior to the experiment. These vaccines were fed at a level of five mg/gm of ration to groups of 100 fall chinook salmon (mean weight 15 gm). The duration of the vaccination feeding period was 15 days followed by a post-immunization period of five days. The animals were then challenged naturally at Lint Slough for 20 days.

Description of Specific Parenteral Vaccination Experiments

Determination of the Effectiveness of Vaccines Prepared from Selected V. anguillarum Isolates

In the in vivo studies concerning the possible antigenic variation between isolates from consecutive years, parenteral, as well as oral, immunization was used. A vaccine was prepared from LS272 and

one from LS173, and these were injected intraperitoneally into groups of 100 fall chinook salmon (mean weight 15 gm). The fish received 0.1 ml of the adjuvant-cell suspension mixture 30 days prior to exposure to challenge at Lint Slough. Mortality data for these fish and a similar group of unvaccinated animals were collected for 20 days.

Comparison of the Effectiveness of Parenteral and Oral Vaccination

An experiment was designed to determine whether parenteral administration of vaccine would provide protection to fish and to compare the efficacy of this method to oral immunization. In these studies two types of fish were used, spring chinook salmon (mean weight 3.8 gm) and fall chinook salmon (mean weight 22.7 gm). Experimental groups consisted of 200 animals in the orally vaccinated groups and 150 fish in the groups which were injected.

The oral bacterin used in this experiment was the lyophilized whole cell vaccine, and it was incorporated into OTD at a level of two mg/gm of ration. The spring chinook were fed 30 gm of diet/200 fish per day, while the fall chinook received 100 gm of diet/200 fish per day. The duration of oral vaccination was 30 days followed by a 15 day post-vaccination period.

Fish which were parenterally vaccinated were injected intraperitoneally with 0.1 ml of the Freund adjuvant-saline suspension

of cells. Both types of fish received the same dosage (approximately 2×10^8 cells/animal) and were vaccinated 50 days prior to the time they were challenged. Throughout this period in fresh water, these animals were maintained on a ration of OTD containing no vaccine.

The orally and parenterally vaccinated fish were simultaneously exposed for 40 days to natural challenge with V. anguillarum. The mortality of these animals was compared to similar groups of fish which had not received any vaccination treatment.

Serological Methods

Preparation of Rabbit Antiserum

Vibrio anguillarum cells were grown on agar slants in eight ounce prescription bottles as previously described. After 24 hours incubation, the cells were removed from the agar surface with 10 ml PBS and washed three times by centrifugation. The pellet from the last washing was resuspended in five ml PBS and mixed with an equal volume of Freund complete adjuvant. One ml of this mixture was injected subcutaneously into each of four sites near the axillary lymph nodes of a New Zealand white rabbit. When an agglutination titer of at least 1:512 was attained, 50 ml of blood were taken from the rabbit's lateral ear vein. The blood was allowed to clot at room temperature and then placed at 4 C overnight for clot retraction. The serum

was harvested after centrifugation, filter sterilized using a 0.45 μm membrane filter, and stored at -26 C. Antisera which was to be used for diagnostic purposes was stored in small amounts using one ml tuberculin syringes in order to avoid repeated freezing and thawing.

Harvesting Fish Serum

Blood was usually collected from fish by severing the caudal artery and allowing the blood to drip into centrifuge tubes or by filling capillary tubes. When larger animals were used, however, blood was collected by cardiac puncture. Blood was left at 4 C overnight and the serum was harvested after centrifugation. Serum not used immediately was stored at -26 C.

Determination of Agglutination Titers

The bacterial antigen for agglutination titers was produced by growing V. anguillarum cells on bottle slants as previously described. The cells were washed three times in PBS by centrifugation and then resuspended to an optical density of 0.85 at 525 nm in a Spectronic 20. The microtiter system (Cooke Engineering, Alexandria, Va.) was employed using the 0.050 ml diluters and drop pipettes. Serial dilutions of the test samples using PBS were made in disposable plates with "U" wells. After addition of the antigen, the plates were incubated at 25 C for two hours and then overnight at 4 C. The results

were read either macroscopically or by using a dissection microscope (20X).

Titration of Vibriocidal Antibody

One of the required elements for the titration of vibriocidal activity is complement. Chiller et al. (1969) has reported that when working with systems involving salmonids, it is necessary to use complement derived from the same or similar family.

At the Wildlife Commission's Roaring River Hatchery, blood from three-year-old male rainbow brood trout was collected in chilled conical centrifuge tubes and allowed to clot. The serum from 50 animals was harvested after centrifugation (600 × g, 10 minutes) and pooled. The pooled serum was shell frozen in a lyophilization flask using dry ice and acetone, and transported to the laboratory in dry ice where it was then lyophilized. The lyophilized serum was powdered and placed in screw cap vials in 0.1 gm quantities (the dry weight of two ml of serum). These samples were stored at -60 C and used as a source of complement from salmonids.

The methods used for the titration of vibriocidal activity were similar to those described by Benenson et al. (1968). The test sample was diluted in tissue culture microplates (Linbro, New Haven, Ct.) using microdiluters and pipette droppers calibrated to deliver 0.025 ml (Cooke Engineering Co., Alexandria, Va.). Dilution

from 1:2 to 1:4,096 were made in PBS. The complement was reconstituted using cold 0.1 percent peptone in saline (0.85 percent NaCl). A dilution of 1:2 of the serum's original volume was made. The complement was mixed 1:1 with a suspension of V. anguillarum cells which had been diluted in PBS. Aliquots of 0.025 ml of the chilled bacterial suspension-complement mixture was added to each of the two-fold serial dilutions of the test samples. The plates were sealed with sealing tape and incubated for one hour at 25 C. After this incubation period, 0.15 ml of BHI was added to each well of the test plates. Controls which were used included: (1) complement-bacterial suspension and BHI, (2) bacterial suspension, test sample and BHI, (3) bacterial suspension, complement and BHI, and (4) bacterial suspension and BHI.

After addition of BHI, the plates were resealed and incubated at 25 C until visible turbidity could be detected in wells containing only the bacterial suspension and BHI. This incubation period was approximately six to eight hours and depended on the number of organisms in the original bacterial suspension. Preliminary observation was made with an inverted microscope (400 X) and final readings were made after overnight refrigeration at 4 C. A clear distinction could be made between the turbid fluid in wells where vibrios had grown and clear fluid in those wells where the organisms had been killed and lysed.

Serum from immunized coho salmon was harvested and tested for the presence of vibriocidal antibodies. Fish were either orally

vaccinated for 15 consecutive days with an OMP ration containing 5 mg of vaccine/gm or parenterally immunized by intraperitoneal injection. Serum from unvaccinated fish was used as a negative control, and the test was also done on rabbit anti-*V. anguillarum* serum.

In a subsequent study serum was collected from fish which had been orally vaccinated by the same regimen. These animals, however, had been exposed to and survived a natural challenge for three months. Included in this experiment were groups of rainbow trout and chinook salmon which had not been vaccinated but had withstood the natural challenge.

RESULTS

Comparison of Various Vaccine Preparations

The original bacterin used as a vaccine for vibriosis consisted of formalin-treated, sonicated, lyophilized V. anguillarum cells (Nelson, 1972). In this experiment various vaccines were prepared and their efficacy compared to the original bacterin. Formalin-killing was omitted in one preparation to determine if that treatment had any adverse effects on the protective antigens of the cell. The necessity of exposing antigens by sonic disruption was tested by preparing a lyophilized whole cell vaccine. After challenge to V. anguillarum total mortality among vaccinated lots of fish ranged from 7 to 11 percent, while the unvaccinated control experienced 70 percent mortality (Table 7). Ten days after initial exposure, increasing mortality had ceased. When these groups of fish had been challenged for 20 days, a new group of control fish was taken to Lint Slough to insure that a valid challenge continued to exist. These animals experienced an 86 percent mortality due to vibriosis after ten days. Results obtained indicated that all methods of vaccination elicited some degree of protection when compared to the unvaccinated control group. These data showed that formalin is not detrimental to protective antigens and that sonication is unnecessary in the preparation of an efficacious vaccine.

Table 7. Efficacy of three oral bacterins for control of vibriosis in spring chinook salmon.

Bacterin administered[a]	Number of fish/group[b]	Total number of deaths[c]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
Formalin-killed Lyophilized Sonicate	200	24	21	11
Formalin-killed Lyophilized Whole Cells	200	14	14	7
Lyophilized Sonicate No Formalin Treatment	200	15	13	7
Unvaccinated Control	200	147	140	70

[a] Fed at a level of 2 mg/gram of Oregon Test Diet for 30 days followed by a 15 day post-vaccination period in fresh water.

[b] Mean weight 3.8 gm/fish.

[c] After 40 days natural challenge to Vibrio anguillarum in salt water.

When in a subsequent study, the immunogenicity of a lyophilized whole cell vaccine was compared to that of a preparation that had not been lyophilized, similar results were obtained (Table 8). The unvaccinated fish died at a level of 55 percent, while the groups of animals which had received the vaccines had mortalities of six and seven percent. This showed that lyophilization is unnecessary in the preparation of an effective immunogen.

Determination of the Effectiveness of Selected Vaccination Periods

When the length of the vaccination period is varied, it appeared that increasing the time of vaccination administration to more than 15 days did not appreciably increase the protection elictied to experimental animals (Table 9). Data collected from experiments in which the vaccination periods were less than 15 days, however, indicated that decreasing the number of dosages did affect the protection provided by the vaccine (Table 10).

Determination of the Effectiveness of Selected Oral Vaccine Concentrations

Preliminary studies on vaccine concentration were done to determine whether increasing the amount/gm of diet given to fish would increase their resistance to vibriosis. Groups of animals which were fed 2, 5, or 10 mg of vaccine/gm of ration experienced mortality due

Table 8. Efficacy of two oral vaccines, lyophilized whole cells and wet packed whole cells, for control of vibriosis in fall chinook salmon.

Bacterin administered	Number of fish/group[c]	Total number of deaths[d]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
Lyophilized Whole Cells[a]	200	19	14	7
Wet-packed Whole Cells[b]	200	16	12	6
Unvaccinated Control	200	115	110	55

[a]Fed at 2 mg/gm of Oregon Test Diet for 45 days.

[b]Fed at 10 mg/gm of Oregon Test Diet for 45 days.

[c]Mean weight 0.3 gm/fish.

[d]After 40 days natural challenge to Vibrio anguillarum in salt water.

Table 9. Efficacy of selected vaccination periods for the control of vibriosis in fall chinook salmon.

Vaccination period[a] (days)	Number of fish/group[b]	Total number of deaths[c]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
15	200	30	21	12
30	200	30	25	13
45	200	41	32	16
0 (Unvaccinated Control)	200	170	168	84

[a]Vaccine fed at a level of two mg of lyophilized whole cell bacterin/gm of Oregon Moist Pellets.

[b]Mean weight 0.3 gm/fish.

[c]After 40 days natural challenge to Vibrio anguillarum in salt water.

Table 10. Efficacy of selected vaccination periods for the control of vibriosis in coho salmon.

Vaccination period[a] (days)	Number of fish/group[b]	Total number of deaths[c]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
15	100	0	0	0
10	100	5	4	4
5	100	7	7	7
2	100	13	12	12
1	100	13	13	13
0 (Unvaccinated Control)	100	27	26	26

[a]Vaccine fed at a level of two mg of wet whole cell bacterin/gm of Oregon Moist Pellets.

[b]Mean weight 15 gm/fish.

[c]After 20 days natural challenge to Vibrio anguillarum in salt water.

to vibriosis of 25, 19, and 18 percent respectively. Sixty-six percent of the unimmunized fish died of vibriosis (Table 11).

Because the results from this experiment indicated that increasing vaccine concentration did not increase protection, a subsequent study was undertaken to test lower vaccine concentrations. All the dosage levels examined in this experiment also provided protection when administered to fish (Table 12). The unvaccinated control group had a 22 percent mortality, while groups receiving 0.5, 1, 2, or 5 mg of vaccine/gm of diet experienced losses of one or two percent, indicating that very low concentrations of antigen were needed to stimulate the immune response if fed over prolonged periods.

Immunization at Selected Water Temperatures

In both experiments in which fish were orally vaccinated at temperatures ranging from 3.9 to 20.6 C, all groups of animals receiving the bacterin were well protected when compared to the unvaccinated control fish. All immunized groups had mortalities of less than five percent while control animals experienced mortalities as high as 83 percent (Tables 13 and 14). The results of the replicated groups in the second experiment demonstrated that not only could fish be effectively immunized at lower water temperatures but also that a very small amount of the immunogen was needed to supply the stimulus for the immune response. For example, the fish vaccinated

Table 11. Efficacy of selected vaccine concentrations for control of vibriosis in fall chinook salmon.

Vaccine concentration administered[a]	Number of fish/group[b]	Total number of deaths[c]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
2	200	55	50	25
5	200	42	37	19
10	200	38	36	18
0 (Unvaccinated Control)	200	139	138	66

[a] Mg of vaccine/gm of Oregon Test Diet fed for 15 days.

[b] Mean weight 6.5 gm/fish.

[c] After 30 days natural challenge to Vibrio anguillarum in salt water.

Table 12. Efficacy of selected vaccine concentrations for the control of vibriosis in coho salmon.

Vaccine concentration administered[a]	Number of fish/group[b]	Total number of deaths[c]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
0.5	100	1	1	1
1.0	100	2	2	2
2.0	100	8	4	4
5.0	100	6	2	2
0.0 (Unvaccinated Control)	100	24	22	22

[a]Mg of vaccine/gm of Oregon Test Diet fed for 15 days in fresh water.

[b]Mean weight 12.6 gm/fish.

[c]After 20 days natural challenge to Vibrio anguillarum in salt water.

Table 13. Efficacy of oral vaccination of coho salmon held at selected water temperature..

Temperatures at which fish were vaccinated [a]	Number of fish/group[b]	Total number of deaths[c]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
3. 9	100	6	0	0
6. 7	100	3	0	0
9. 5	100	4	0	0
12. 2	100	9	1	1
15. 0	92	19	5	5
17. 8	100	8	0	0
20. 6	100	5	1	1
12. 2 (Unvaccinated Control)	100	44	37	37

[a]Vaccinated with 5 mg of vaccine/gm of Oregon Moist Pellets for 15 days followed by a 14 day tempering period.

[b]Mean weight 6. 5 gm/fish.

[c]After 20 days natural challenge to Vibrio anguillarum in salt water.

Table 14. Efficacy of oral vaccination of coho salmon held at selected water temperatures.

Temperature at which fish were vaccinated[a]	Number of fish/group[b]	Total number of deaths[c]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
3.9	75	2	1	1
	100	13	5	5
6.7	95	4	2	2
	60	4	1	2
9.5	97	0	0	0
	100	6	2	2
12.2	90	2	0	0
	80	6	0	0
15.0	96	1	1	1
	100	0	0	0
17.8	97	0	0	0
	86	1	0	0
20.6	99	0	0	0
	87	0	0	0
12.2 (Unvaccinated Control)	100	72	72	72
	100	86	83	83

[a]Vaccinated with 5 mg of vaccine/gm of Oregon Moist Pellets for 15 days followed by a seven day tempering period.

[b]Mean weight 3.3 gm/fish.

[c]After 20 days natural challenge to Vibrio anguillarum in salt water.

at 3.9 C were offered only 3,000 μg of the wet packed vaccine during the 15 day vaccination period.

Comparison of the Effectiveness of Vaccines Prepared from Selected V. anguillarum Isolates

Vaccines prepared from bacterial cultures originating in the Pacific Northwest (Lint Slough, Lummi, and Manchester) seemed to be more effective than European strains (Italy and Denmark) when immunized fish were exposed to the challenge at Lint Slough (Table 15). Although better protection was elicited by vaccines from Pacific Northwest isolates, some degree of resistance was also offered to fish immunized with European strains when the mortality was compared to an unvaccinated group of animals. The mortalities of the different groups were: Lint Slough, 11 percent; Lummi, 20 percent; Manchester, 2 percent; Italy, 36 percent; Denmark, 52 percent; and unvaccinated controls, 86 percent. This indicated that there were some protective antigens which all the organisms possessed in common; however, it also showed that there may be some antigenic differences among the different isolates.

It was of interest to determine whether strains of V. anguillarum isolated in different years would provide a similar degree of protection when used in vaccine preparation. If antigenic changes occur from year to year, these changes may preclude the use of a standard

Table 15. Efficacy of oral vaccines prepared from selected isolates of Vibrio anguillarum for the control of vibriosis in fall chinook salmon.

Isolate from which bacterin was prepared[a]	Number of fish/group[b]	Total number of deaths[c]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
Lint Slough	100	44	11	11
Lummi	100	51	20	20
Manchester	100	12	2	2
Italy	100	65	36	36
Denmark	100	68	52	52
Unvaccinated Control	100	96	86	86

[a] Vaccinated with 5 mg wet whole cell bacterin/gm of Oregon Test Diet.

[b] Mean weight 15 gm/fish.

[c] After 20 days natural challenge to Vibrio anguillarum at Lint Slough.

bacterial culture for vaccine preparation. The groups of animals receiving LS272 bacterins had slightly higher mortalities (11 and 16 percent) than the group fed the LS173 vaccine (one percent). The unvaccinated controls experienced 52 percent mortality (Table 16). These experiments suggested bacteria isolated from different years did yield adequate protection when used as vaccines.

Determination of the Effectiveness of Parenteral Vaccines Prepared from two *V. anguillarum* Isolates

Vaccines were prepared from two Lint Slough isolates derived from epizootics in consecutive years. These experiments were done to determine if protective antigens vary from year to year. The results showed that an equal degree of protection was elicited by each vaccine. The studies also demonstrated that intraperitoneal injection of the vaccine was an effective method of controlling vibriosis (Table 17). The vaccinated animals had mortalities of one and two percent while unimmunized controls experienced a loss of 52 percent.

Comparison of the Effectiveness of Parenteral and Oral Vaccination

Groups of fall and spring chinook salmon were vaccinated both orally and by intraperitoneal injection. Each vaccinated group showed little variation in mortality. The immunized spring chinook showed

Table 16. Efficacy of orally administered vaccines prepared from two Lint Slough isolates for the control of vibriosis in fall chinook salmon.

Bacterin administered[a]	Number of fish/group[b]	Total number of deaths[c]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
LS272 (prepared 1972)	100	17	16	16
LS272 (prepared 1973)	98	17	11	11
LS173 (prepared 1973)	100	1	1	1
Unvaccinated Control	100	52	52	52

[a] Vaccinated with 5 mg of vaccine/gm of Oregon Test Diet for 30 days.

[b] Mean weight 15 gm/fish.

[c] After 20 days natural challenge to Vibrio anguillarum in salt water.

Table 17. Efficacy of parenterally administered vaccines prepared from two Lint Slough isolates.

Bacterin administered[a]	Number of fish/group[b]	Total number of deaths[c]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
LS272	99	3	1	1
LS173	100	5	2	2
Unvaccinated Control	100	52	52	52

[a] Bacterin was administered by intraperitoneal injection; each fish received 2×10^8 cells suspended in saline and Freund adjuvant.

[b] Fall chinook salmon mean weight 15 gm/fish.

[c] After 20 days natural challenge to Vibrio anguillarum in salt water.

losses of three percent in the injected group and four percent in the orally immunized lot. The unimmunized control animals had a 72 percent mortality (Table 18). Similar results were obtained from the experiment using fall chinook. Injected fish had a seven percent mortality; the orally vaccinated group showed a ten percent mortality, and the control animals experienced an 80 percent loss (Table 19). These results indicated that both methods of vaccine administration can be effective in the control of vibriosis.

Titration of Vibriocidal Antibodies in Parenterally or Orally Immunized Fish

Although serum agglutinating titers were not detected in either groups of fish used in the first experiment, the presence of vibriocidal antibodies was determined. The serum titers from both orally and parenterally immunized fish were 1:16 (Table 20). Anti-*V. anguillarum* serum prepared in rabbits gave a much higher titer of 1:512.

When vibriocidal antibody titers were examined in fish exposed to *V. anguillarum*, it was found that not only serum from vaccinated, but also unvaccinated animals possessed vibriolytic activity (Table 21). These animals probably produced the antibodies after contact with the organism in salt water and possibly after a subclinical case of vibriosis. The serum from all groups of fish examined showed titers of 1:16 or 1:32.

Table 18. Comparison of the efficacy of parenteral and oral administration of vaccine for the control of vibriosis in spring chinook salmon.

Method of vaccine administration	Number of fish/group[c]	Total number of deaths[d]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
Fed Orally[a]	200	11	7	4
Injected Intraperitoneally[b]	150	5	4	3
Unvaccinated Control	200	147	144	72

[a]Vaccine was dry whole cells incorporated into Oregon Test Diet and fed at a level of 2 mg of vaccine/gm of diet for 30 days.

[b]Vaccine was 0.1 ml of a Freund adjuvant-saline suspension containing 2×10^8 cells.

[c]Mean weight 3.8 gm/fish.

[d]After 40 days natural challenge to Vibrio anguillarum in salt water.

Table 19. Comparison of the efficacy of parenteral and oral administration of vaccine for the control of vibriosis in fall chinook salmon.

Method of vaccine administration	Number of fish/group[c]	Total number of deaths[d]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
Fed Orally[a]	200	26	19	10
Injected Intraperitoneally[b]	150	13	10	7
Unvaccinated Control	200	172	160	80

[a] Vaccine was dry whole cells incorporated into Oregon Test Diet and fed at a level of 2 mg of vaccine/gm of diet for 30 days.

[b] Vaccine was 0.1 ml of a Freund adjuvant-saline suspension containing 2×10^8 cells.

[c] Mean weight 23.0 gm/fish.

[d] After 40 days natural challenge to Vibrio anguillarum in salt water.

Table 20. Vibriocidal antibody titers of coho salmon which had been orally or parenterally immunized against Vibrio anguillarum.

Serum sample	Antibody titer 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Oral	+[a]	+	+	+	-[b]	-	-	-	-	-
Injected	+	+	+	+	-	-	-	-	-	-
Rabbit anti-V. anguillarum	ag[c]	ag	ag	ag	ag	ag	+	+	+	-
Control	-	-	-	-	-	-	-	-	-	-

[a] Indicates lysis of the cells.

[b] Indicates growth of the cells.

[c] Indicates agglutination of the cells.

Table 21. Vibriocidal antibody titers of orally vaccinated fall chinook salmon and rainbow trout which had survived natural challenge with Vibrio anguillarum.

Serum sample	Antibody titer 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
Vaccinated Rainbow	+[a]	+	+	+	+	-[b]	-	-	-	-	-	-
Vaccinated Rainbow	+	+	+	+	-	-	-	-	-	-	-	-
Vaccinated Chinook	+	+	+	+	+	-	-	-	-	-	-	-
Vaccinated Chinook	+	+	+	+	+	-	-	-	-	-	-	-
Non-vaccinated Chinook	+	+	+	+	-	-	-	-	-	-	-	-
Non-vaccinated Chinook	+	+	+	+	-	-	-	-	-	-	-	-
Rabbit anti-V. anguillarum	ag[c]	ag	ag	ag	ag	+	+	+	+	+	+	+
Unexposed Control Chinook	-	-	-	-	-	-	-	-	-	-	-	-

[a] Indicates lysis of the cells.

[b] Indicates growth of the cells.

[c] Indicates agglutination of the cells.

Studies with vibriocidal antibody detection in fish are in the preliminary stages of development and should be more exhaustively studied. However this technique does offer potential for an in vitro method for the detection of the immune response in orally vaccinated fish. This would enable the immune state of animals to be measured by some criterion other than death.

DISCUSSION

These studies explored oral and parenteral immunization as potential methods for the control of vibriosis in salmonid fish reared in marine aquaculture facilities. Salmonids are well-suited for immunization against V. anguillarum because they can be vaccinated in fresh water, where the disease organism does not occur, and then transferred to salt water where vibriosis is endemic. Using this system, effective oral and parenteral vaccines were developed and several parameters for their use were examined.

In the preparation of the original vaccine against V. anguillarum, the bacteria were killed using formalin and then sonically disrupted to expose as many cellular antigens as possible. The sonicated bacteria were then lyophilized to facilitate weighing and storage. In an effort to produce the bacterin more economically, experiments were conducted to determine the necessity of these two steps. Disruption of the bacterial cells was omitted and lyophilized whole cells were used effectively, demonstrating that sonication did not increase the availability of the protective antigens. Subsequently it was shown that lyophilization was also an unnecessary step. Therefore an effective preparation for the immunization of fish against V. anguillarum can be produced simply by growing bacterial cells in liquid media, killing them, and harvesting by centrifugation. The preparation of the immunogen has been condensed to the simplest and most economical

method possible. Although lyophilization is not required for an efficacious vaccine, it may be desirable in some situations to facilitate storage and shipping of the preparation.

There are several methods that can be used to kill bacteria for use in vaccines. Two of these are heat killing and the use of bactericidal chemicals such as formalin, phenol or chloroform. In preparation of the bacterin described here, formalin (0.3 percent) was the agent employed. To test whether this chemical was detrimental to protective antigens, a bacterin was prepared which used sonication to render the bacteria non-viable. It was demonstrated that, when the preparation was compared to a formalin-killed bacterin, this chemical had no measurable adverse effects on the protective antigens. Other workers exploring oral vaccines for different fish pathogens have reported that killing with phenol or chloroform instead of formalin may produce more effective vaccines (Schaperclaus, 1974; Anderson and Ross, 1972). Still other investigators advocate heat-killing. Killing V. anguillarum by these three methods should be examined as possible means for increasing the potency of the vaccine.

In addition to experiments involving preparation of the bacterin, several studies were done concerning improvement of the effectiveness of the vaccine. Altering the amount of bacterin administered and determining the limits under which the preparation elicits protection

to fish were studied. These studies were approached from two aspects. First, to examine the length of time the bacterin was fed and second, to ascertain the effect selected concentrations of vaccine in the diet had on protection.

Decreasing the number of days the oral vaccine was administered from 45 to 10 days did not seem to affect the protection elicited. Although feeding the bacterin for one, two, or five days did afford some degree of protection, these shorter periods of administration did result in higher mortality when compared to groups of fish which had been fed the vaccine for at least ten days.

Experiments concerning vaccine concentration demonstrated that increasing the amount of vaccine does not increase the amount of protection afforded fish receiving the bacterin. In subsequent studies, lower vaccine concentrations were examined. Although the lower limit was not reached, it was found that as little as 0.5 mg wet weight of vaccine/gm of diet fed for 15 days was capable of providing protection.

Decreasing the number of days the vaccine is fed and the concentration administered will make the preparation more economical to use. However, it must be stressed that all the investigations described here were conducted with experimental lots of 100 or 200 fish and that these conditions differ from those on the production scale. Small groups of fish can be fed under somewhat controlled

conditions that enable most animals in the population to receive their portion of vaccine. This type of control is not feasible on the production level, and insuring that animals are immunized may necessitate longer feeding periods and higher concentrations of vaccine than the minimums described.

Several investigators have explored the effect of temperature on the immune response of poikilothermal animals. Each of those experiments was performed by injecting the immunogen and then following the appearance of agglutinating or precipitating antibodies. The studies described here are unique in that the vaccine was administered orally and the immune response was measured by the degree of protection provided when the animals were exposed to a natural challenge. Although agglutinating antibodies could not be detected, animals vaccinated at each of the temperatures employed were protected against V. anguillarum. In the preliminary experiment, fish were tempered in six days to 12 C and held at that temperature for seven days before being challenged in salt water. It could be argued that the immune response was elicited during this time at a favorable temperature. In an attempt to circumvent this problem, a second study was done, and after the vaccination period the fish were held at their respective temperatures for seven days, tempered to 12 C during a six day period and then challenged immediately. The results from both studies demonstrated that oral immunization against

vibriosis can be successfully accomplished at a wide range of water temperatures. Therefore, effective oral vaccination is not precluded by temperature situations found at diverse hatchery locations and at various times of the year where water temperature may be as low as 4 C or as high as 21 C.

Pacha and Kiehn (1969) studied the antigenic composition of 15 V. anguillarum isolates. Ten of the isolates were from the Pacific Northwest and derived from epizootics in salmonid species. Two were isolated from Pacific herring, and the remainder were European strains from either finnock or codling. These investigators divided the organisms into three serotypes: one consisting of those isolates derived from Pacific Northwest salmonid species, one including the organisms isolated from Pacific herring, and the other comprised of the European isolates. Although the antigenic composition was not the same for all strains, there were some antigens which each serotype possessed in common.

Because vibriosis is a worldwide problem it was of interest to do preliminary studies to determine whether the protective antigens were the same or different in selected isolates from different areas of the world. Several bacterins were prepared and evaluated at Lint Slough. It was found that isolates derived from epizootics of salmonids in the Pacific Northwest provided the greatest degree of protection. Strains of V. anguillarum from Europe elicited some immunity.

The isolate derived from a salmonid from Italy gave intermediate protection, and the strain from Denmark isolated from codling, afforded a decreased degree of resistance. These data indicated that although there are differences, each strain possesses some common protective antigens.

If alterations in protective antigens occur frequently, vaccine production from one strain of the bacterium would not be feasible. Because previous experiments indicated that some antigenic differences occur among V. anguillarum strains, an in vivo experiment was done to determine if these variations occurred in Lint Slough isolates which were derived from epizootics in two consecutive years. Fish were vaccinated with bacterins prepared from the two strains. Both vaccines elicited a similar degree of resistance; this was especially evident when they were administered intraperitoneally. Although it appeared that there are antigenic differences among isolates from diverse areas of the world, these results indicated that this type of variation does not exist in organisms from a defined area. The tests done using various V. anguillarum isolates indicate that employing a standardized isolate is not feasible in production of vaccine for immunization of fish in diverse areas. It is feasible, however, to prepare efficacious bacterins from one strain of V. anguillarum if the vaccinated animals are exposed to vibriosis where the original strain was isolated. Antigenic analyses should be done to confirm the

in vivo tests described. Serotyping of *V. anguillarum* could facilitate decisions concerning the effectiveness of bacterins prepared from various strains of the organism.

The use of a specific strain of *V. anguillarum* for vaccine production would enable continual production of vaccine without requiring a recent isolate of the bacterium. However, it would be advantageous to know if the preparation loses potency during long storage. Experiments done demonstrated that the wet whole cell bacterin did not lose its effectiveness when stored for one year at -26 C. The lyophilized vaccine preparations have also been shown to retain their immunogenicity after extended periods of time (Nelson, 1972).

Most of the studies described here involved the development of orally administered vaccines. Some experimentation was done with parenteral bacterins, and this method of vaccine administration was shown to be effective for the control of vibriosis. Although intraperitoneal injection is not as convenient a method as feeding for vaccine administration, it is still a feasible alternative, and does offer some advantages over oral immunization. One is that vaccination of each animal can be assured. Another is that parenteral immunization is believed to provide longer lasting protection. Experiments have been done which indicated that the protection elicited by oral vaccination wanes after approximately three months if the animals remain unchallenged (Garrison, 1972). This type of experimental

data is unavailable for fish parenterally vaccinated, but it is known that agglutinating antibody titers remain high after three months if the vaccine is administered with an adjuvant. However, Krantz et al. (1963),working with an A. salmonicida vaccine, demonstrated that without an adjuvant, antibody titers can return to pre-immunization levels three months after maximum titers have been reached. A third advantage of parenteral vaccination is that it may require less bacterin. Although the lower limit of antigen needed for oral vaccination has not been determined, at the levels presently prescribed, intraperitoneal injection requires less vaccine. Further investigation concerning dosage with the oral preparation may make it more economical since this method requires no additional step in the rearing of fish. In addition to the inconvenience of parenteral injection of large numbers of fish, the handling of these animals in the manner required for injection can be very detrimental to them. Therefore, although there are certain advantages with parenteral immunization, oral vaccination of fish on a production scale would seem to be the most acceptable method if each technique provides a similar degree of protection.

Throughout these studies an attempt was made to determine the nature of resistance provided to immunized fish by the vaccine. Serum agglutinating antibody titers can be detected in fish vaccinated by intraperitoneal injection, however this type of response was not

observed in orally immunized animals. Experiments were done which suggest that complement-mediated bactericidal activity could be part of the protective mechanism provided by vaccination. The mechanisms responsible for the immune response need to be more exhaustively studied and may include exploring the possibility of secretory antibodies, increased phagocytic activity, or cellular immunity. The detection of these systems has numerous pitfalls, especially when working with such small animals, however this type of experimentation could yield valuable information in determining the reasons for the success or failure of oral vaccines against fish pathogens.

An effective vaccine has been developed for the control of vibriosis in salmonid fish. This bacterin has been extensively examined in laboratory experiments but remains to be tested successfully on a production scale. Laboratory studies are necessarily done under controlled conditions, and such a degree of control is not feasible on a larger scale. Other aspects of the disease process become factors on the production level. Disease resistance depends on the interaction of the hosts, the pathogen, and the environment. Since fish are very dependent on their environment, it can place many stresses on the animals and thus make them more susceptible to disease. Environmental stresses include elevated temperatures, vast temperature fluctuations, low dissolved oxygen levels and high

ammonium levels. Chronic or subclinical infection with disease producing agents is another factor which may lower the resistance of an animal. Because immunity to infection is a relative state and does not insure absolute resistance, any of these, or other unfavorable conditions for the host, can cause the immune state to be overwhelmed and allow the disease process to manifest itself.

It can be observed that even on the experimental level, there is not a 100 percent survival among the vaccinated groups. In addition to the possibility of environmentally stressed animals, a portion of the population may be immunologically incompetent. Unlike the evaluation of human or other animal vaccines, this bacterin was tested in a manner which insured that every fish in the vaccinated population was exposed to the causative agent. For these reasons some mortality must be expected among fish vaccinated against vibriosis.

If mariculture is to meet its potential as a source of protein food for man, infectious diseases of fish, and especially vibriosis, must be controlled. Although there are inherent problems in the immunization of fish on a production scale, the vaccine developed during these studies offers a potentially successful method for the control of vibriosis in marine or estuarine environments.

SUMMARY AND CONCLUSIONS

1. Formalin-killed lyophilized whole cells of V. anguillarum were shown to be an effective oral vaccine against vibriosis in salmonid fish.
2. Formalin-killed wet-packed whole cells of V. anguillarum were shown to be an effective oral vaccine against vibriosis in salmonid fish.
3. Vibriosis in salmonid fish was controlled by intraperitoneal injection of killed V. anguillarum cells incorporated into Freund complete adjuvant.
4. Increasing the number of consecutive days the oral vaccine was administered from 10 to 45 days did not increase the degree of protection elicited.
5. Feeding the oral vaccine for less than ten days did not provide as high a degree of protection as longer feeding periods.
6. Concentrations ranging from 0.5 to 10 mg wet weight of vaccine per gm of ration were effective in controlling vibriosis.
7. Fish can be orally immunized against vibriosis at water temperatures ranging from 3.9 to 20.6 C.
8. Wet whole cell vaccines can be stored at -26 C for one year without losing potency.

9. Although there are antigenic similarities among V. anguillarum isolates, it may not be feasible to prepare vaccines from a standardized strain if the bacterins are to be used in diverse areas of the world.
10. Vaccines used in a defined area (e.g. the Pacific Northwest) may be prepared from a standardized strain of V. anguillarum isolated from that location.
11. Agglutinating antibodies can be detected in parenterally vaccinated fish but not in animals which are orally immunized.
12. Complement-mediated bactericidal antibody may be one of the protective mechanisms stimulated by oral immunization.
13. Fish as small as 0.3 gm are immunologically competent and can be orally immunized.
14. Vaccination of fish does not afford the animals absolute protection against vibriosis.
15. By elimination of sonication and lyophilization, the vaccine can be prepared by growing the cells, killing them and then harvesting. This is possibly the most economical method available for production of the bacterin.

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