MFR PAPER 1290

Immunization of Salmonids for Control of Vibriosis

J. L. FRYER, J. S. ROHOVEC, and R. L. GARRISON

ABSTRACT—Experimental work done with the immunization of chinook salmon, Oncorhynchus tshawytscha, *and coho salmon,* O. kisutch, *against* Vibrio anguillarum *is described. Experiments include: 1) A comparison of the efficacy of oral and injected bacterins; 2) a study of the protection provided by three selected oral bacterin concentrations; 3) an investigation of three selected periods of bacterin administration; and 4) a study of the effects of diverse water temperatures on oral immunization. Studies with the* V. anguillarum *bacterin indicate that under experimental conditions fish can be immunized against vibriosis.*

Work on fish pathology at Oregon State University has included the development of several experimental immunogens including those of *Flexibacter columnaris, Aeromonas salmonicida,* infectious hematopoietic necrosis virus, and *Vibrio anguillarum.* Experiments with these bacterins and vaccines have been directed toward studying the immune response in Pacific salmon (genus *Oncorhynchus*) and steelhead trout, *Salmo gairdneri.* The primary objective of this research has been potential immunization of these animals for control of infectious diseases which have affected large-scale fish culturing attempts. Efficacious bacterins have been reported for the control of vibriosis in salmonids (Hayashi et al., 1964; Fryer et al., 1972). To date, the most successful immunogen tested in our laboratory has been prepared from cells of *V. anguillarum.*

One of the simplest methods for delivery of bacterins to large fish populations is by oral administration. Therefore, the major portion of this investigation (begun in 1968) has involved developing oral bacterins and testing conditions under which they can be effectively used. In addition to oral immunogens, a parenteral administered bacterin was also studied.

J. L. Fryer and J. S. Rohovec are with the Department of Microbiology, Oregon State University, Corvallis, OR 97331. R. L. Garrison is with the Oregon Department of Fish and Wildlife, Research Division, Corvallis, OR 97331.

METHODS

Preparation of Bacterins

The preparation of each antigen is outlined in Figure 1. The procedure for mass culturing of the bacterial cells was the same in the preparation of the oral bacterins. Ten ml of brain heart infusion (BHI) broth (Difco)[1] were inoculated with organisms from a stock culture of *V. anguillarum.* After 12 hours incubation at 25°C, 2 ml of this broth culture were used to inoculate two separate 1-liter quantities of broth which were also incubated 12 hours at 25°C. These two separate liters of culture were then used as the inoculum for 30 liters of medium which had been sterilized in a fermentor. After an incubation period of 10-12 hours at 28°C, 500 ml of a 20 percent dextrose solution was added and the culture allowed to incubate an additional 10-12 hours.

For the preparation of the wet-packed, whole cell bacterin, the bacterial cells were killed in the fermentor by the addition of 100 ml Formalin solution directly into the 30 liter broth culture. The cells were harvested after 1 hour, frozen, and stored at −26°C.

To prepare lyophilized whole cells, the cells were harvested by high speed, continuous-flow centrifugation. Two hundred fifty grams of the harvested cells were then resuspended in 1 liter of a saline solution containing 0.3 percent Formalin and mixed for 24 hours at 25°C. The cells were then lyophilized.

Cells were also prepared for parenteral administration. Because smaller quantities of the bacterin were needed for this type of immunization, cells were cultured on BHI agar surfaces. This was done by preparing agar slants in 8-ounce prescription bottles which were inoculated with 1 ml of a 12 hour culture of the desired organism. After incubation (24 hours at 25°C), the cells were removed from the agar surface with 0.3 percent Formalin-saline solution. Bacterial cells remained in this solution for 1 hour and were then washed three times by centrifugation in phosphate buffered saline.

Bacterin Administration and Challenge

The method for oral vaccination followed a similar procedure in all experiments conducted. Fish, either chinook salmon, *O. tshawytscha,* or coho salmon, *O. kisutch,* were fed a diet containing the bacterin incorporated at a selected level per gram of ration. Diets used in this study were either Oregon Moist Pellets (OMP) (Hublou, 1963) or Oregon Test Diet (OTD) (Lee et al., 1967). This diet was administered to fish held at a freshwater facility supplied with pathogen-free well water at an ambient temperature of 12°C. It was also possible to vary the water temperature in experimental aquariums from 4° to 23°C.

Efficacy of the bacterins was tested by exposing experimental fish to a natural challenge of *V. anguillarum* in a saltwater rearing impoundment at Lint Slough on the Oregon coast. Groups of animals were held in 1-m diameter fiberglass tanks which were furnished

[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

with saltwater. *Vibrio anguillarum* is endemic at this rearing impoundment and reaches epizootic proportions in the warmer months of the year when the water temperature rises to 12°C or above (Cisar and Fryer, 1969).

A necropsy was performed on all experimental animals which died during the challenge. The animals were examined for gross pathological symptoms, dissected aseptically, and bacteriological cultures were then prepared from kidney tissue on BHI agar. After incubation, the plates were examined for typical colonies of *V. anguillarum*. Presumptive tests included microscopic examination using the gram reaction, and morphology and motility by means of phase contrast microscopy. Recovery of *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.

Comparison of Oral and Injected *Vibrio* Bacterins

An experiment was designed to determine whether parenteral administration of bacterin would provide protection to fish and to compare the efficacy of this method to oral immunization. Experimental groups of fall chinook salmon consisted of 200 fish in the orally vaccinated groups and 150 fish in the injected groups. The oral bacterin consisted of lyophilized whole cells incorporated into OTD at a concentration of 2 mg/g of ration. The fish received 100 g of diet/200 fish per day. The bacterin feeding period was 30 days followed by a 15 day postvaccination period. Fish which were injected intraperitoneally received approximately 2×10^8 cells/animal in 0.1 ml of a Freund adjuvant-saline suspension 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 containing no bacterin. 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 a similar group of fish which had received no bacterin.

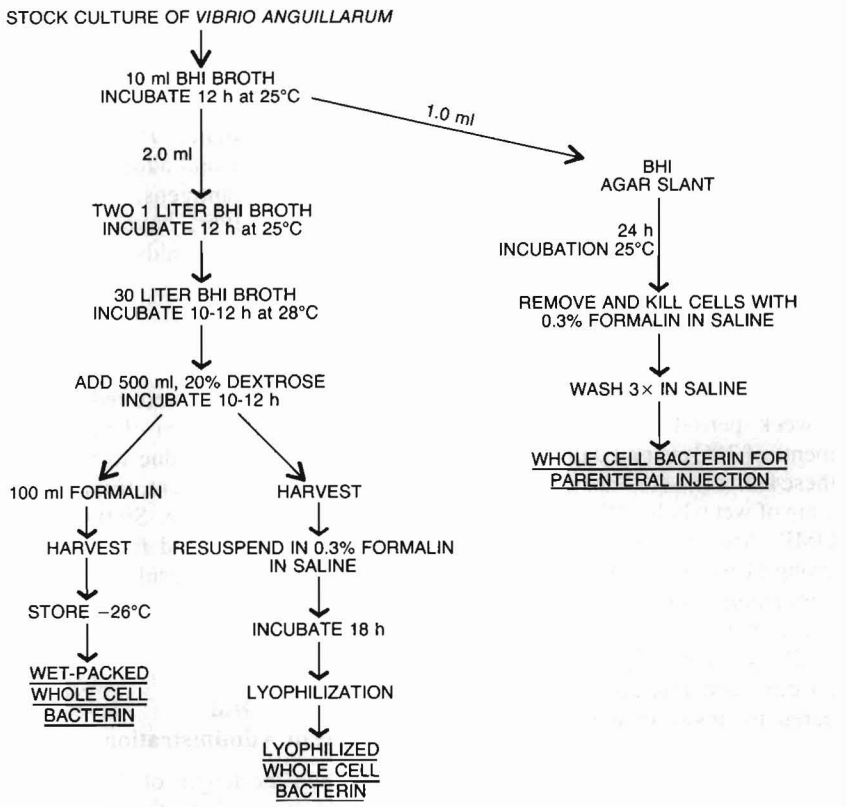

Figure 1.—Methods used for *Vibrio anguillarum* bacterin preparation.

Concentration of Oral Bacterin

Early experiments routinely employed either 5 or 10 mg of vaccine per gram of diet. These levels were arbitrarily selected and no comparative information was available. In order to gain some understanding of the concentration required to produce immunity with the orally administered bacterins, the following experiment was designed. Groups of 200 fall chinook salmon (mean weight 6.5 g) were fed wet-packed whole cell bacterin at levels of 2, 5, or 10 mg/g of OTD for 30 days. After this feeding period, the fish remained in fresh water for 15 days and were then challenged at Lint Slough for 30 days.

Length of Oral Bacterin Administration

There was also interest in the length of time that the vaccine containing rations should be administered to produce increased protection. The lyophilized whole cell bacterin was added to OMP at a level of 2 mg/g and fed to groups of 200 fall chinook salmon (mean weight 0.8 g). One group of animals received this diet for 15 days, a second for 30 days, and a third for 45 days. The vaccination periods were begun so that each of the three experimental groups received its last bacterin dose on the same day. All vaccinated fish and an unvaccinated control group were then maintained in fresh water for 15 days before being simultaneously challenged for 40 days at Lint Slough.

Effects of Temperature on Oral Immunization

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 poikilothermic

animals is slower at lower temperatures (Muroga and Egusa, 1969; Avtalion et al., 1973; Paterson and Fryer, 1974). Because all of the immunization experiments that have been previously described here had been conducted at water temperatures of 12°C, it was desirable to determine what effect diverse water temperatures had on the immune response of orally vaccinated animals.

Replicate groups of coho salmon (mean weight 3.3 g) were acclimated to each of seven temperatures: 3.9°, 6.7°, 9.5°, 12.2°, 15.0°, 17.8°, and 20.6°C. Acclimation of fish took place during a 1-week period at temperature increments of 3°C every 2 days. For 15 days these animals were fed a diet containing 2 mg of wet whole cell bacterin/gram of OMP. After this feeding period, each group of fish was held at its respective temperature for 1 week before being reacclimated to 12°C. The fish were challenged naturally at Lint Slough for 20 days and mortality was then compared to unvaccinated animals which had been held at 12°C throughout the freshwater phase of the experiment.

RESULTS

Comparison of Oral and Injected *Vibrio* Bacterins

Each group of fish vaccinated either orally or intraperitoneally showed little variation in mortality 40 days after challenge (Table 1). Injected fish had a 7 percent mortality, the orally vaccinated group 10 percent, and the control animals experienced an 80 percent loss. These results indicated that both methods of vaccine administration were effective for the control of vibriosis.

Prior to challenge, serum samples from fish of each vaccinated group were tested for the presence of agglutinating antibody. Animals which had been immunized parenterally had high antibody titers (1:640); however, fish which were orally immunized did not show detectable levels of agglutinating antibody. This indicated that different immune responses are stimulated by each of these two methods of vaccination. Similar observations have been made by Schaperclaus (1972). Although the mechanism of protection has not been determined for orally immunized fish, cellular immunity or secretory antibody may be involved. Fletcher and White (1973) reported the presence of secretory antibody in the intestines of plaice, *Pleuronectes platessa* L., after oral administration of *V anguillarum* antigens. These experiments, however, have not been repeated using salmonids as experimental animals.

Concentrations of Oral Bacterins

The fish which were fed 2, 5, or 10 mg of bacterin/gram of ration experienced mortalities (due to vibriosis) of 25, 19, and 18 percent, respectively, 30 days after challenge. Sixty-six percent of the unimmunized fish died of vibriosis (Table 2). Results indicated that fish can be protected with a bacterin concentration of as low as 2 mg/g of ration.

Length of Oral Bacterin Administration

When the length of the vaccination period was varied, it appeared that increasing the time of administration to more than 15 days did not appreciably increase the protection provided these experimental animals (Table 3). Fish vaccinated for 15, 30, and 45 days experienced mortalities of 12, 13, and 16 percent, respectively, 40 days after challenge, while the unvaccinated control group had an 84 percent mortality.

Effects of Temperature on Oral Immunization

The results of this experiment indicate that diverse water temperatures do not preclude oral immunization with bacterins prepared for the control of *V. anguillarum*. All immunized groups had mortalities of less than 5 percent, 20 days after challenge. The control animals experienced losses as high as 83 percent (Table 4).

DISCUSSION

Throughout our research with the *V. anguillarum* immunogens, the efficacy of both oral and injected bacterins has appeared feasible. Since these studies indicate that under experimental conditions fish can be immunized against vibriosis, the application of these techniques to aquaculture seems possible. However, to our knowledge, no definitive production trial has been conducted, and at the present time these products do not exist in a licensed form available to the aquaculture industry. We believe that before the pharmaceutical industry begins to prepare immunogens for licensing and use by fish culturists, seven major problems must be considered.

Table 1.—Comparison of the efficacy of parenteral and oral administration of bacterin for the control of vibriosis in fall chinook salmon.

Method of bacterin administration	No. of fish/ group[1]	Total no. of deaths[2]	No. of deaths caused by vibriosis	Mortality caused by vibriosis (%)
Fed[3]	200	26	19	10
Injected intraperitoneally[4]	150	13	10	7
Unvaccinated control	200	172	160	80

[1]Mean weight 23.0 g/fish.
[2]After 40 days natural challenge to *Vibrio anguillarum* in saltwater.
[3]Bacterin was dry, whole cells incorporated into Oregon test diet and fed at a level of 2 mg of vaccine/gram of diet for 30 days.
[4]Bacterin was 0.1 ml of a Freund's adjuvant-saline suspension containing 2×10^9 cells.

Table 2.—Efficacy of selected bacterin concentrations for control of vibriosis in fall chinook salmon.

Bacterin concentration administered[1]	No. of fish/ group[2]	Total no. of deaths[3]	No. of deaths caused by vibriosis	Mortality caused by vibriosis (%)
2	200	55	50	25
5	200	42	37	19
10	200	38	36	18
Unvaccinated control	200	139	132	66

[1]Milligrams of bacterin/gram of Oregon test diet fed for 15 days.
[2]Mean weight 6.5 g/fish.
[3]After 30 days natural challenge to *Vibrio anguillarum* in saltwater.

Table 3.—Efficacy of selected vaccination periods for control of vibriosis in fall chinook salmon.

Vaccination period[1] (days)	No. of fish/ group[2]	Total no. of deaths[3]	No. of deaths caused by vibriosis	Mortality caused by vibriosis (%)
15	200	30	21	12
30	200	30	25	13
45	200	41	32	16
Unvaccinated control	200	170	168	84

[1]Bacterin fed at a level of 2 mg of lyophilized whole cell bacterin/gram of Oregon moist pellets.
[2]Mean weight 0.8 g/fish.
[3]After 40 days natural challenge to *Vibrio anguillarum* in saltwater.

Table 4.—Efficacy of oral immunization of coho salmon held at selected water temperatures.

Temperature[1] (°C)	No. of fish/ group[2]	Total no. of deaths[3]	No. of deaths caused by vibriosis	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
Unvaccinated control	100	72	72	72
	100	86	83	83

[1]Vaccinated with 5 mg of vaccine/gram of Oregon moist pellets for 15 days followed by a 7-day acclimating period.
[2]Mean weight 3.3 g/fish.
[3]After 20 days natural challenge to *Vibrio anguillarum* in saltwater.

1) There appear to be difficulties in moving from controlled laboratory experiments with 200-500 fish to full-scale production. Experimental results may vary with large numbers of fish in the production situation, and what is possible experimentally may not be directly applicable to aquaculture as it now exists.

2) Serotyping of fish pathogens is in its infancy, and the possibility of large numbers of serotypes of these pathogens could complicate the production of bacterin. Careful selection of bacteria or viruses for vaccine preparation must be made.

3) As techniques of immunology begin to be applied more extensively in aquaculture, there will be a real need to reemphasize the definition of immunity. There is a tendency to consider immunity as a shield built around the animals which makes them permanently invulnerable to a particular pathogen. Immunity is not permanent invulnerability. It is a relative state of insusceptibility brought on by one of three conditions: a) Having the disease and recovering; b) having a subclinical case of the disease; or c) being artificially immunized. The protection afforded by a particular immunogen is relative, and it can be overcome by any of several factors such as environmental stress, overwhelming presence of the pathogen, or the appearance of a different serotype. Therefore, progress in immunization of fish offers aquaculture increased protection from fish pathogens but not necessarily the eradication of disease.

4) Since environmental stress is a factor which influences susceptibility, and because fish are particularly vulnerable to these stresses, the impact of environmental alterations on the immune state needs further study. We have, on one occasion, seen an abrupt change in temperature produce sufficient stress in previously immune animals to cause them to become susceptible to the pathogen.

5) As work in both fish pathology and aquaculture continues, the development of reproducible artificial challenge systems will become imperative.

6) There is a need to develop methods for measuring immunity in fish other than direct exposure to the specific pathogen with subsequent evaluations based on death or survival.

7) Finally, novel methods for vaccine delivery should be explored. Although injection and oral administration of bacterins are capable of eliciting immune responses, each method poses inherent difficulties when dealing with large populations of fish.

ACKNOWLEDGMENTS

The authors express their appreciation to J. L. Zinn for technical assistance rendered during these experiments, and for his continued interest in the project.

This investigation was conducted under a contract with the Oregon Department of Fish and Wildlife and financed in part by Anadromous Fish Act Funds (PL 89-304) through the U.S. Fish and Wildlife Service.

The material contained in this report was originally submitted in a thesis by J. S. Rohovec in partial fulfillment of the requirements for the Ph.D. degree in Microbiology, Oregon State University. Material contained in this report has also been presented at two international symposia (Rohovec et al., 1975; Fryer et al. 1976) and is included here in order to provide completeness of the proceedings.

LITERATURE CITED

Avtalion, R. R., A. Wojdani, Z. Malik, R. Shahrabani, and M. Duczminer. 1973. Influence of environmental temperature on the immune response in fish. Curr. Top. Microbiol. 61:1-35.

Cisar, J. O., and J. L. Fryer. 1969. An epizootic of vibriosis in chinook salmon. Bull. Wildl. Dis. Assoc. 5:73-76.

Fletcher, T. C., and A. White. 1973. Antibody production in plaice (*Pleuronectes platessa* L.) after oral and parenteral immunization with *Vibrio anguillarum*. Aquaculture 1:417-428.

Fryer, J. L., J. S. Nelson, and R. L. Garrision. 1972. Vibriosis in fish. *In* R. W. Moore (editor), Progress in fishery and food science. 5:129-133. Univ. Wash. Publ. Fish., New Ser., Seattle.

Fryer, J. L., J. S. Rohovec, G. L. Tebbit, J. S. McMichael, and K. S. Pilcher. 1976. Vaccination for control of infectious diseaes in Pacific salmon. Fish Pathol. 10:155-164.

Hayashi, R., S. Kobayashi, T. Kamata, and H. Ozaki. 1964. Studies on the vibrio-disease of rainbow trout (*Salmo gairdneri irideus*) II. Prophylactic vaccination against the vibrio-disease. J. Fac. Fish. Prefect. Univ. Mie. 6:181-191.

Hublou, W. F. 1963. Oregon pellets. Prog. Fish-Cult. 25:175-180.

Lee, D. S., J. N. Roehm, and R. O. Sinnhuber. 1967. Effect of three fatty acids on the growth of rainbow trout (*Salmo gairdneri*). J. Nutr. 92:93-97.

Muroga, K., and S. Egusa. 1969. Immune response of the Japanese eel to *Vibrio anguillarum*. I. Effects of temperature on agglutinating antibody production in starved eels. Bull. Jap. Soc. Sci. Fish. 35:868-874.

Paterson, W. D., and J. L. Fryer. 1974. Effect of temperature and antigen dose on the antibody response of juvenile coho salmon (*Oncorhynchus kisutch*) to *Aeromonas salmonicida* endotoxin. J. Fish. Res. Board Can. 31:1743-1749.

Rohovec, J. S., R. L. Garrison, and J. L. Fryer. 1975. Immunization of fish for the control of vibriosis. Proc. Third U.S.-Japan Meeting on Aquaculture, Tokyo, Japan, October 15-16, 1974, p. 105-112. Jpn. Fish. Agency, and Jpn. Sea Reg. Fish. Res. Lab., Niigata.

Schaperclaus, W. 1972. Orale und parenterale aktive immunisierung von karpfen gegen *Aeromonas punctata*. Archiv Exp. Veterinaermed. 26(5):863-874.

MFR Paper 1290. From Marine Fisheries Review, *Vol. 40, No. 3, March 1978. Copies of this paper, in limited numbers, are available from D822, User Services Branch, Environmental Science Information Center, NOAA, Rockville, MD 20852. Copies of* Marine Fisheries Review *are available from the Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402 for $1.10 each.*