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Spray Vaccination: A Method for the Immunization of Fish[1]

R. W. GOULD,* P. J. O'LEARY,*** R. L. GARRISON,**
J. S. ROHOVEC,*** and J. L. FRYER***

*National Fisheries Research Center, U. S. Fish and Wildlife Service, Seattle, Washington, 98115 (U.S.A.)
**Oregon Department of Fish and Wildlife, Research Division, Corvallis, Oregon, 97330 (U.S.A.)
***Department of Microbiology, Oregon State University, Corvallis, Oregon, 97331 (U.S.A.)

An economical, efficacious vaccine delivery system for immunizing fish has been developed which employs a liquid spray apparatus operated at pressures up to 7.0 kg/cm^2 (0 to 100 lb/in^2). A bacterin consisting of formalin-killed *Vibrio anguillarum* culture was both antigenic and immunogenic when sprayed on coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Salmo gairdneri*). The technique, referred to as spray (shower) vaccination, was found to confer higher levels of immunity against virulent *V. anguillarum* than oral vaccination.

Introduction

The use of immunizing agents is emerging as a complement to other methods of disease control and has been used successfully both experimentally and commercially (FRYER *et al.*, 1977). Two problems exist in the development of fish vaccines: first, to provide efficacious preparations; and second, to provide economic methods for mass vaccination. Vaccines for fish have been delivered by several methods to include: parenteral injection; orally, through incorporation of vaccines into the animal's diet; hyperosmotic infiltration, by placing fish in a hyperosmotic solution followed by a vaccine bath; direct immersion into vaccine suspensions; or by direct addition of vaccine to water in which fish are held (CORBEL, 1975; AMEND, 1976; AMEND and FENDER, 1976; SCHACTE, 1976; ANTIPA and AMEND, 1977; CROY and AMEND, 1977; FRYER, *et al.*, 1977). Each of these techniques has its inherent advantages and disadvantages. Although intraperitoneal injection appears to be most effective, this method is time consuming and stresses the fish being vaccinated. Oral administration is perhaps the most desirable method of vaccine delivery, but in some cases has not provided high levels of resistance (GUNNELS, *et al.*, 1976). Hyperosmotic infiltration and direct immersion are used to vaccinate small fish but may not be economical with larger animals. Addition of vaccine to water has been used experimentally only with an attenuated viral vaccine (FRYER *et al.*, 1976).

This report describes another method for mass immunization of fish. A bacterin against *Vibrio anguillarum* was administered by spraying fish with antigens prepared by selected methods. This technique provided a fast efficacious means of administering vibrio bacterin.

Materials and Methods

Experimental Animals

The Oregon Department of Fish and Wildlife (ODFW) supplied coho salmon (*Oncorhynchus kisutch*) from their Research Division, Fall Creek Hatchery and Cascade Hatchery; rainbow trout (*Salmo gairdneri*) were obtained from Wizard Falls Hatchery. Experimental fish were maintained in fiberglass tanks supplied with 12°C fish pathogen free well water at the Oregon State University Fish Disease Laboratory, Corvallis, Oregon.

Experimental design required the marking of animals to identify different treatment groups. Fish were marked using a brass

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branding probe cooled in dry ice and acetone or with fluorescent dyes administered by spraying under pressure (6.3 to 7.0 kg/cm^2). Fish were identified by the brand or dye color under an ultraviolet light.

Preparation of Bacterins

All bacterins were prepared from *V. anguillarum* (serotype I) isolated from moribund coho salmon reared in Lint Slough, Waldport, Oregon. Cultures of this organism (LS 1–74) were maintained in the lyophilized state and were grown in Brain Heart Infusion (BHI) broth (Difco) or on BHI agar (Difco). For bacterin production the organism was cultured in broth for 96 h at 30°C. After incubation the bacteria were killed by overnight exposure to 0.3% formalin or heat inactivated by autoclaving at 121°C for 15 min. The killed bacterial cells, used as bacterin, were harvested by centrifugation and left in the wet-packed state or lyophilized. The entire culture suspension was also employed as a bacterin. Preparation and use of the oral bacterin has been described (ROHOVEC, *et al.*, 1975).

Bacterin Administration

Bacterins were administered in three ways: orally, by immersion, or by spraying. In these experiments wet-packed whole cell bacterin was incorporated into Oregon Moist Pellets (HUBLOU, 1963) at a level of 5 mg of bacterin per g of diet and fed for 45 days. Vaccination by immersion was accomplished by placing fish into the undiluted bacterin suspension for a period of one minute or less. Fish were spray vaccinated at a pressure of 6.3 to 7.0 kg/cm^2 utilizing a commercial sand blasting gun with a venturi-feeding reservoir containing bacterin. Experimental groups of fish were placed in a dip net and sprayed 5 to 10 sec with the tip of the spray apparatus positioned 20 to 25 cm from the fish. The animals were moved in the net so that each received direct application of bacterin on some portion of the body surface. An atomizer was used in a similar manner to spray vaccinate at zero pressure.

Artificial Challenge

An artificial water borne *V. anguillarum* challenge was developed to test the efficacy of bacterins which were administered to fish by selected methods. Marked experimental groups were transferred to 93 *l* stainless steel raceway challenge tanks receiving 12°C water at approximately 6 *l*/min. The water temperature was increased from 12°C to 18°C over a period of 1 h. The viable count of an 18-h broth culture of *V. anguillarum* was estimated photometrically and a standard plate count was performed to determine the exact viable bacterial concentration. The water flow to the tanks was stopped and sufficient inoculum of the challenge culture was added to achieve a final concentration of approximately 1×10^6 bacteria per m*l* of water. After 15 min the 18°C water flow was resumed. Mortality was recorded daily and dead fish were examined bacteriologically and the causative agent was isolated and identified using serological techniques.

Detection of Serum Agglutinating Antibody

After severing the caudal artery fish were bled into capillary tubes for collection of individual serum samples. The blood was allowed to clot at room temperature and was placed at 5°C overnight for clot retraction. Serum was harvested after centrifugation (500×g for 15 min) and used immediately or stored at −26°C. Antibody titers were determined by the microtiter method using 0.025 m*l* diluters and drop pipettes and "U" bottom microtiter plates (Cooke Engineering, Alexandria, Virginia). The diluent was 0.01 molar phosphate buffered saline (PBS) and the antigen was *V. anguillarum* cells which had been washed 3 times by centrifugation (3000× g for 20 min) in PBS and resuspended in PBS to an optical density of 0.85 at 525 nm on a Bausch and Lomb Spectronic 20. Antigen was prepared fresh for each set of antibody determinations. After two fold serial dilutions of serum and addition of antigen, plates were incubated for 1 h at room temperature and then at 5°C overnight. Titers were read as the last dilution that displayed agglutination when viewed at 20× magnification with a dissecting microscope.

Results

Lyophilized whole cell bacterin was mixed

with an inert carrier at selected concentrations ranging from 100 to 0.001 mg bacterin per g of carrier. This was prepared as a thick slurry and sprayed onto coho salmon (mean weight 15 g). After 24 days, 5 fish from each experimental group were bled and serum agglutinating antibody titers determined. The remaining fish were challenged with *V. anguillarum*. Results presented in Table 1 show that all levels of bacterin tested except 0.001 mg/g produced an agglutinating antibody response, although the titers induced by levels below 1 mg/g were slight. Solid protection was elicited by levels equal to or greater than 1 mg bacterin per g of carrier.

An experiment was performed to determine whether bentonite at a concentration of 0.15% had an adjuvant effect when administered with the formalin-killed cultures. Thirty days after vaccination, 5 fish (mean weight 23 g) which received bacterin with bentonite had agglutinating antibody titers with a geometric mean of 1: 388. The fish vaccinated with bacterin only had a geometric mean titer of 1: 32 and unvaccinated control animals did not respond.

Selected air pressures required to immunize fish using the spray technique were compared with the immersion method. Bacterins were prepared from a heat-killed or formalin-killed 96-h culture. One group of rainbow trout (mean weight 15 g) was spray immunized at 6.3 kg/cm^2 and another at 0 kg/cm^2. A spray atomizer was used to approximate zero pressure. A third group was immersed in bacterin for 60 sec and a fourth group was immersed for approximately 2 sec. Forty-four days after vaccination all groups were tested for agglutinating antibody titer and for resistance to challenge with *V. anguillarum*. All vaccinated groups were protected even though antibody titers were low (Table 2). Control groups experienced mortality of 72 and 88%.

Table 1. Comparison of agglutinating antibody and protection against virulent *Vibrio anguillarum* in coho salmon sprayed with selected concentrations of bacterin mixed with an inert carrier

Concentration (mg bacterin/g carrier)	Geometric mean[a] agglutinating antibody titer^{-1}	Number of fish challenged[b]	Number of deaths	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis[c]
0.001	5	23	15	13	57[d]
		22	11	11	50[d]
0.01	21	22	17	15	68[d]
		23	17	16	70[d]
0.1	21	22	14	14	64[d]
		23	11	11	48[d]
1	32	29	2	2	7
		21	1	1	5
10	147	21	0	0	0
		23	0	0	0
100	147	21	0	0	0
		24	0	0	0
No Bacterin	2	25	18	18	72[d]
		22	19	19	86[d]

[a] Obtained from titers of five individual fish.

[b] The waterborne challenge level was 2.0×10^6 bacteria per m*l*.

[c] Percent mortality equals deaths caused by vibriosis divided by number of fish challenged minus non-specific deaths.

[d] The differences between the mean control mortality and mean 0.001 mg/g and 0.1 mg/g mortality are significant at the 95 percent confidence level, $\chi^2 = 5.39$ and 5.61, respectively. The difference between the mean control mortality and 0.01 mg/g level is not significant at the 95 percent confidence level, $\chi^2 = 0.29$.

Table 2. Comparison of agglutinating antibody and protection against virulent *Vibrio anguillarum* in rainbow trout both sprayed and immersed in heat-killed or formalin-killed bacterin

Treatment	Method for bacterial inactivation	Mean geometric[a] agglutinating titer^{-1}	Number of fish challenged[b]	Number of deaths	Number of deaths due to vibriosis	Percent mortality caused by vibriosis[c]
Spray (6.3 kg/cm^2)	formalin	36	25	0	0	0
			25	0	0	0
	heat	18	25	0	0	0
			25	0	0	0
Spray (0–1 kg/cm^2)	formalin	32	25	0	0	0
			25	0	0	0
	heat	9	25	0	0	0
			25	0	0	0
Immersion (2 sec)	formalin	28	25	0	0	0
			25	0	0	0
	heat	6	25	0	0	0
			25	0	0	0
Immersion (60 sec)	formalin	7	25	0	0	0
			25	0	0	0
Control	—	0	25	18	18	72
			25	22	22	88

[a] Obtained from titers of five individual fish.
[b] The waterborne challenged level was 5.12×10^6 bacteria per m*l*.
[c] Percent mortality equals deaths caused by vibriosis divided by number of fish challenged minus non-specific deaths.

Table 3. Comparison of agglutinating antibody and protection against virulent *Vibrio anguillarum* in coho salmon both orally and spray vaccinated

Treatment	Mean geometric[a] agglutinating titer^{-1}	Number of fish challenged[b]	Number of deaths	Number of deaths due to vibriosis	Percent mortality due to vibriosis[c]
5 mg bacterin per	0	23	4	4	17
g OMP plus		16	4	4	25
placebo spray		15	4	4	27
Spray vaccinated	147	20	0	0	0
		16	0	0	0
		20	1	0	0
5 mg bacterin per	78	17	0	0	0
g OMP plus spray		20	0	0	0
vaccinated		20	2	2	10
Control: placebo spray	0	20	11	11	55
vaccinated		23	15	15	65
		20	11	11	55

[a] Obtained from titers of ten individual fish.
[b] Waterborne challenge was 6.1×10^5 bacteria per m*l*.
[c] Percent mortality equals death caused by vibriosis divided by number of fish challenged minus non-specific deaths.

Efficacy of spray vaccination and oral administration of bacterin was compared. Coho salmon (mean weight 30 g) received oral bacterin (5 mg bacterin per g diet) for 45 days. A similar group of fish was spray vaccinated with formalin-killed bacterin. A third lot of fish was vaccinated using both techniques. Thirty-five days after vaccination, the animals were artificially challenged with *V. anguillarum* and agglutinating antibody titers were determined in samples of fish from each group. Results (Table 3) indicate that a higher degree of immunity was conferred by spray vaccination than by oral administration.

Discussion and Conclusions

Vibrio anguillarum serotype I offers a good experimental model for testing methods for immunizing fish: first, because bacterins prepared from this organism are very antigenic and very small amounts are required to elicit an immune response in fish (GOULD, 1977) and second, because a reproducible artificial challenge which simulates natural exposure and infection has been developed. Using this model, spray vaccination, a new method for mass immunization of fish, was developed and some parameters under which this technique can be used were studied.

In the original tests an inert carrier was incorporated with the bacterin and it was determined that a relatively small antigenic mass was necessary to elicit an immune response in fish. Bentonite, a known adjuvant, was also examined as a carrier for the bacterin. In one test, bacterin with bentonite stimulated the production of higher agglutinating antibody titers than bacterin without bentonite. These data indicate that bentonite may have some adjuvant effect when used with the spray vaccination technique. However, no comparision was made on the ability of these two bacterins to elicit resistance to challenge and it is felt that it is premature to relate antibody titers to degree of protection.

Experiments performed comparing the efficacy of bacterins administered with and without pressure and by immersion indicate that pressure is not required to induce an immune response in the fish. Heat and formalin-killed bacterins were also compared. No differences in efficacy could be ascertained because all groups of vaccinated fish were solidly protected. From these experimental results it was impossible to determine which method of bacterin inactivation or administration was superior.

Although there are distinct advantages to oral vaccination of fish, on occasion the immunity elicited is low and the resistance is short lived (GUNNELS, 1976; GARRISON, 1972). It was found that spray vaccination offered a higher degree of immunity than oral immunization. Although the experiments reported here were carried out over a short period of time; tests still in progress indicate that the duration of immunity offered by spray vaccination is in excess of 400 days.

Spray vaccination may also have some advantages over immersion methods. Immersion types of vaccination have been recommended for fish of relatively small sizes. Spray vaccination can be used at any stage of the fish's life cycle in which the animal is immunocompetent and presents fewer logistic problems when immunizing large fish. This method could be employed at any time when fish are moved within a rearing facility and might conceivably be adopted to existing fish pumps, eliminating any additional handling stress placed on the animals.

The development of the spray vaccination technique offers another alternative for the mass immunization of fish. This method has been demonstrated to be effective when vaccinating fish with *V. anguillarum* bacterins and some of the parameters for its use have been studied. Further refinement of this method is in progress and similar studies should be performed with other fish pathogens.

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噴霧ワクチン——魚類免疫の一方法

魚類を免疫する経済的かつ効果的なワクチン投与法として 0～7.0 kg/cm² 圧力の液体噴霧器を用いる方法が開発された。*Vibrio anguillarum* のホルマリン死菌で作られたバクテリンをギンザケ (*Oncorhynchus kisutch*) とニジマス (*Salmo gairdneri*) に噴霧したところ，抗原性および免疫原性が認められた。

噴霧ワクチン (spray vaccination) と呼ばれるこの技法は病原菌 *Vibrio anguillarum* に対して経口ワクチン (oral vaccination) より高い水準の免疫を賦与することができることが判明した。