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Effects of immersion in live *Vibrio anguillarum* and simultaneous oxytetracycline treatment on protection of vaccinated and non-vaccinated rainbow trout *Salmo gairdneri* against vibriosis

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ABSTRACT: One-year old rainbow trout *Salmo gairdneri* were challenged with *Vibrio anguillarum*, Serotype 1, in a 2-stage experiment. During the first stage, lots of dip-vaccinated and of non-vaccinated (control) fish were challenged using bath exposure. Approximately half of the challenged lots were given an 8 d oral treatment with oxytetracycline at the time of challenge; the remaining lots were untreated. Thirty d after the first challenge, survivors of the first exposure, as well as lots of previously non-challenged dip-vaccinated and control trout, were subjected to a second bath challenge. No fish were medicated following the second challenge. Groups of vaccinates and controls surviving from the initial challenge showed significantly lower mortality rates following the second challenge than did their previously non-challenged cohorts. The effects of previous exposure and vaccination on mortality rate were additive. In addition, medication during the first challenge did not significantly affect mortality rates observed during the second challenge, in either vaccinated or control trout.

INTRODUCTION

Vibriosis in fish has been described in peracute, acute and chronic forms (Richards & Roberts 1978). In Sweden, vibriosis most frequently occurs as an acute disease, affecting salt and brackish water pen-reared 1 yr old rainbow trout *Salmo gairdneri* and hatchery-reared fingerling rainbow trout. Early treatment for 7 to 10 d with oral oxytetracycline (OTC) generally limits mortalities. Recurrent outbreaks of vibriosis among treated fish within one growing season are, however, not uncommon. According to Swedish National Veterinary Institute records, 5 of 13, 8 of 19 and 9 of 26 vibriosis-affected farms in 1983, 1984 and 1985, respectively, were required to medicate trout 2 or more times at greater than 3 wk intervals. The reasons behind this phenomenon are not known. Egidius & Andersen (1979) have suggested that antibiotic treatment of early outbreaks may contribute to a more or less chronic form of the disease.

It is generally believed that a mild natural challenge with *Vibrio anguillarum* serves to stimulate fish's immune processes (Evelyn & Ketcheson 1980). The intensity and duration of the natural exposure likely influence the degree of its immunizing effect. The amount of protection gained by survivors of a vibriosis outbreak or by populations exposed to natural low (non-epizootic) levels of *V. anguillarum* is not established.

This paper reports the results of an aquarium challenge experiment conducted with *Vibrio anguillarum*, Serotype 1. One purpose of this study was to examine whether exposure to virulent *V. anguillarum* would affect the mortality rate of trout following a subsequent challenge. A second objective was to investigate whether medication applied during an initial challenge would adversely affect the potentially immunizing effect of that challenge. Tests were conducted in both vaccinated and non-vaccinated rainbow trout.

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MATERIALS AND METHODS

An experiment consisting of 2 sequential challenges was performed using 42 g yearling rainbow trout obtained from a commercial freshwater hatchery. Approximately 170 fish were netted from the delivered group and dip-vaccinated by direct immersion for 20 s in a 10-fold dilution of a divalent commercial vibriosis bacterin. Following vaccination, vaccinates and approximately 200 non-vaccinates were maintained in separate freshwater tanks at 13 to 14 °C.

A bath challenge with virulent *Vibrio anguillarum*, Serotype 1, originating from frozen (−70 °C) ampules of a stock culture isolated from moribund rainbow trout, was used in all tests. Bacteria from the frozen ampules were re-cultured for 24 h at 24 °C on blood agar, suspended in physiologic saline, and diluted to desired concentrations immediately prior to addition to the challenge baths. General challenge procedures followed were: (1) cultures were prepared as described above, then diluted to appropriate concentrations using a 0.9 % NaCl solution; (2) fish were immersed in the challenge suspension for 15 min; (3) fish were returned from the challenge bath to flowing freshwater aquaria; (4) dead fish were removed twice daily and at least half of the dead fish were cultured for the challenge organism. Kidney material was streaked on blood agar and isolates were identified with rabbit anti-*V. anguillarum* serum by the rapid slide agglutination test.

The water temperatures and challenge dosages used are shown in Table 1. Water temperature was maintained at 13 to 14 °C during the the first ('low-dose') challenge (Challenge A) and was elevated to 16 to 18 °C over the 2 d prior to and during the second ('high-dose') challenge (Challenge B).

Challenge A was conducted 26 d after vaccination. In Challenge A, one group (2 lots) of vaccinated fish and one group (2 lots) of non-vaccinated fish were medicated orally with OTC for 8 d at a dosage of 75 mg OTC (kg fish)$^{-1}$ d^{-1}. Medication was initiated 6 h pre-challenge. Non-medicated cohorts (2 lots of vaccinates and 3 lots of controls) were also included in Challenge A. No medication was used in Challenge B.

Fish surviving the first challenge ('exposed' fish) remained under observation in their aquaria for 30 d following exposure, at which time they were re-challenged (Challenge B), as shown in Table 1. Previously non-exposed vaccinated and non-vaccinated fish remaining in the holding tanks were transferred to aquaria 8 d prior to the second challenge; they were challenged at the same time and according to the same procedure as 'exposed' survivors. The observation period following the second challenge continued for 18 d (7 d following the last recorded mortality).

Two ([1] and [2]) 2-way analyses of variances (Neter & Wasserman 1974) were performed (at $\alpha = 0.05$). We tested for the presence of main effects of [1] previous exposure alone (Table 2) and [2] medication in conjunction with previous exposure (Table 3) on the mortality rates observed in Challenge B. The importance of interaction effects of vaccine status with [1] exposure and [2] medication was also investigated.

Table 1. *Salmo gairdneri*. Experimental design for and mortality rates observed in *Vibrio anguillarum* (VA), Serotype 1, bath challenges of 1 yr old rainbow trout

Challenge	Challenge dosage (VA ml^{-1})	Water temp. (°C)	Groups included[1] (no. lots per group)[2]	% mortality[3]
A	1×10^4	13–14	VEm(2)	0(0)
			VEM(2)	0(0)
			CEm(3)	11(3)
			CEM(2)	0(0)
B[4]	1×10^7	16–18	VEm(2)	2(3)
			VEM(2)	7(3)
			Ve(2)	13(12)
			CEm(2)	57(12)
			CEM(2)	43(17)
			Ce(2)	96(0)

[1] V: vaccinated by immersion; C: 'control', i.e. non-vaccinated; E: 'exposed', i.e. fish included in Challenge A, with survivors re-challenged in B; e: 'non-exposed', i.e. fish first infected in Challenge B; M: medicated with oxytetracycline for 8 d during Challenge A; m: not medicated during Challenge A

[2] No. of lots = number of aquaria per group; 24 to 25 fish and 20 to 24 fish (dependent on number of survivors from Challenge A) were used in each aquarium in Challenges A and B, respectively

[3] Percentages shown are means (SD) of all lots included in each group, with each lot weighted as one

[4] Challenge B was conducted 30 d after Challenge A

Table 2. *Salmo gairdneri*. Effects of (1) vaccination with bacterin and (2) bath exposure to live *Vibrio anguillarum*, Serotype 1, (VA) on mortality rates in 1 yr old rainbow trout following VA bath challenges

Day 1: Vaccination	Day 25: Challenge A	Days 55–73: Challenge B mortality rates
Vaccinated	Exposed vaccinated (VEm)	2 %
	Non-exposed vaccinated (Ve)	13 %
Non-vaccinated	Exposed control (CEm)	57 %
	Non-exposed control (Ce)	96 %

Summary of analyses of variance[1] (significance tests conducted at $\alpha = 0.05$)
(1) Vaccination with bacterin provides strong protection against vibriosis. (Average mortality rate in vaccinates, VEm and Ve [7.5 %], was significantly lower than that in controls, CEm and Ce [76.5 %])
(2) Vaccination by exposure to live pathogens provides strong protection against vibriosis. (Average mortality rate in exposed fish, VEm and CEm [29.5 %], was significantly lower than that in non-exposed fish, Ve and Ce [54.5 %])
(3) Protective effects of vaccination by exposure to bacterin and to live pathogens are additive. (Relative percent survival[2] of exposed vaccinates, VEm *vs* CEm [96.5 %], did not differ significantly from that of non-exposed vaccinates, Ve *vs* Ce [86.5 %])

[1] Arcsin transformations of mortality rates were used in the ANOVA

[2] Relative percent survival $= 1 - \left(\frac{\%\text{ mortality in fish receiving treatment in question}}{\%\text{ mortality in fish not receiving treatment in question}} \right) \times 100$

RESULTS

The following notation in Table 1 is used to identify treatment groups: (1) 'V', vaccinated or 'C', control; (2) 'E', exposed in Challenge A or 'e', first exposed in Challenge B; (3) 'M', medicated or 'm', non-medicated during Challenge A. An 'M' or 'm' label is only applied to 'E' groups, i.e. those groups included in Challenge A.

Table 1 presents the average mortality rates observed in the challenge tests. *Vibrio anguillarum* was re-isolated in pure culture from all dead fish sampled during the challenge period.

The results of the statistical analyses can be summarized as follows: (1) immersion vaccination with bacterin provided strong protection against vibriosis (Tables 2 & 3); (2) bath exposure to a low dosage of live pathogens provided strong protection against vibriosis (Table 2); (3) the decrease in mortality rate observed in 'exposed' vaccinated fish, relative to 'exposed' control fish, equalled the decrease in mortality rate observed in non-exposed vaccinated fish, relative to non-exposed control fish; i.e. the effects of bacterin and exposure on mortality rates were additive (Table 2); (4) the 8d course of oral OTC begun just prior to the first

Table 3. *Salmo gairdneri*. Effects of (1) vaccination with bacterin and (2) medication applied at the same time as bath exposure to live *Vibrio anguillarum*, Serotype 1, (VA) on mortality rates in 1 yr old rainbow trout following VA bath challenges

Day 1: Vaccination	Day 25: Challenge A	Medication during Challenge A	Days 55–73: Challenge B mortality rates
Vaccinated	Exposed vaccinated	Medicated exposed vaccinated (VEM)	7 %
		Non-medicated exposed vaccinates (VEm)	2 %
Non-vaccinated	Exposed control	Medicated exposed control (CEM)	43 %
		Non-medicated exposed control (CEm)	57 %

Summary of analyses of variance[1] (significance tests conducted at $\alpha = 0.05$)
(1) Vaccination with bacterin provides strong protection against vibriosis. (Average mortality rate in vaccinates, VEM and VEm [4.5 %], was significantly lower than that in controls, CEM and CEm [50 %])
(2) Medication applied during the first challenge period does not adversely affect the immunizing value of the challenge. (Average mortality rate in medicated fish, VEM und CEM [25 %], did not differ significantly from that in non-medicated fish, VEm and CEm [29.5 %])

[1] Arcsin transformations of mortality rates were used in the ANOVA

challenge did not adversely affect the immunizing value of that challenge, in either vaccinated or non-vaccinated fish (Table 3).

The mortality rates listed in Table 2 also show that vaccination with bacterin only (Ve, 13% mortality rate) provided significantly ($p < 0.05$, Student's t-test) better protection in Challenge B than did 'vaccination' by exposure only (CEm, 57 % mortality rate).

DISCUSSION

This study revealed that exposure to live pathogens reduced mortalities during a second challenge. All vaccinated fish, all medicated control fish and vitually all (89 %) of the non-medicated control fish survived the first challenge (A). Therefore, the lower mortality rates observed for these survivors on second challenge (B) can be ascribed to an immune response and are not due to a 'weeding out' of weaker susceptibles by Challenge A.

Non-vaccinated trout which had survived one low-dose exposure (CEm) had a significantly lower average mortality rate than did non-exposed non-vaccinated (Ce). This result agrees with Braaten & Hodgin's (1976) finding that contact with live *Vibrio anguillarum* cells stimulates immunity (in steelhead trout). It is noteworthy, however, that the average mortality rate of previously exposed controls (CEm) was significantly higher than that of previously non-exposed vaccinated fish (Ve). In other words, a 20 s exposure to the formalin-killed vaccine, at a concentration of approximately 10^9 organisms ml^{-1}, produced substantially higher levels of immunity than did a 15 min exposure to live pathogens, at a concentration of approximately 10^4 organisms ml^{-1}. O'Neill (1979) described a similar dose dependency of the primary immune response of trout. It is questionable, therefore, whether the immunity elicited in non-vaccinated farmed trout from natural challenge with low doses of the vibrio pathogen is sufficient to adequately protect fish from subsequent exposure under more severe conditions (higher pathogen dosages, greater pathogen virulence and/or harsher environmental conditions), if such occur. This elicited immunity is especially unlikely to suffice if the duration and magnitude of the immunizing exposure has been shortened by antibiotic therapy.

Trout which were both dip-vaccinated and exposed to live pathogens in the initial low-dose bath challenge (VEm) died, in the subsequent challenge, at a significantly lower rate than did non-exposed vaccinated trout (Ve). These results should be considered with respect to controversy centering around the existence of anamnesis in salmonids and other fish. O'Neill (1979) and Anderson et al. (1982) demonstrated immunological memory in rainbow trout exposed to DNP conjugates and in brown trout exposed to MS2 bacteriophage, respectively. Harrell (1978) and Andersen & Dixon (1980), on the other hand, did not find a heightened secondary immune response in coho salmon naturally exposed to vibriosis outbreaks and rainbow trout flush exposed to *Yersinia ruckeri* O-antigen, respectively. Studies have shown that environmental factors, such as temperature (O'Neill 1980), and intrinsic factors, such as antigen dose, its timing and route of administration (Rijkers et al. 1980), and antigen carrier characteristics (Anderson et al. 1982), influence the immune response in fish. Anderson & Dixon (1980) suggested that 'further proof [on immunological memory in fish] will rest with protection tests'. The protection tests of the present study demonstrated a heightening of immunity in vaccinates following additional exposure. This corroborates the existence of an anamnestic response in rainbow trout.

The immunizing value, discussed above, of the second exposure in vaccinates, and of the primary exposure in controls, was not adversely affected by the simultaneous application of antibiotic therapy. Previously exposed and medicated fish (CEM and VEM) died at the same rates following a second challenge as did previously exposed, but non-medicated fish (CEm and VEm, respectively). The OTC treatment did not interfere with either vaccinates' or non-vaccinates' ability to respond immunologically to the pathogen, as measured by survival. Medication had an obvious therapeutic effect (in control fish) during the first challenge (A), and appeared to have no immunosuppressive effect. This finding conflicts with those of Anderson et al. (1984), who reported that OTC has direct negative effects on the immune systems of rainbow trout.

We initiated medication early (pre-challenge) in this experiment. Fish farmers usually, however, start feeding OTC only after vibriosis is established in the population. The rationale for our early treatment was to ensure therapeutic levels in individual fish during the earliest stages of infection. Such treatment would more likely affect immune responses than would medication initiated 1 or 2 d after challenge. Even with this early treatment we found, however, no negative effect.

The possible role that antibiotic treatment plays in recurrent vibriosis outbreaks within a given fish farm requires further investigation. The explanation for this phenomenom probably lies in the dynamics of the infection rather than in the hypothesized direct negative effect of OTC on the immune system of the fish. For instance, it is unlikely, under natural conditions, that all fish in a farmed population become simultaneously infected with vibrio (Horne 1982). After initial infection of a few individuals, contact infection plays an impor-

tant role in the spread of the infection within a holding facility (Hastein 1975). Properly applied medication tends to halt the spread of the infection within a holding facility, so that a large portion of fish may actually remain non-exposed. The latter then remain susceptible to a subsequent challenge.

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