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IMMUNIZATION OF SALMONIDS AGAINST *YERSINIA RUCKERI*: SIGNIFICANCE OF HUMORAL IMMUNITY AND CROSS PROTECTION BETWEEN SEROTYPES

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ABSTRACT: Brook trout (*Salvelinus fontinalis*) were immunized with bacterins containing either Serotype 1 or 2 isolates of *Yersinia ruckeri* to determine the relative degree of cross-protection afforded when the fish were challenged with the homologous or heterologous serotype. While fish immunized with pH-lysed bacterins produced highly specific agglutinins that did not cross-react with antigens derived from a heterologous serotype of *Y. ruckeri* all fish were protected against experimental challenge, regardless of which serotype was used for bacterin production and experimental challenge. Other experiments indicated that brook trout injected intraperitoneally with highly specific antibodies could not be passively immunized against experimental challenge.

Key words: *Yersinia ruckeri*, enteric redmouth disease, immunization, circulating antibody, brook trout, *Salmo fontinalis*.

INTRODUCTION

Enteric redmouth (ERM) disease caused severe disease among many hatchery populations of rainbow trout (*Salmo gairdneri*) cultured in the western United States (Ross et al., 1966). Investigations confirmed that these epizootics were caused by *Yersinia ruckeri*, a member of the family Enterobacteriaceae (Ewing et al., 1978). Because of its incidence in the western United States, ERM was considered to have a restricted range until further investigators found that the bacterium was widespread throughout the United States (Bullock et al., 1978), Canada (Wobeser, 1973), Australia (Bullock et al., 1977), and Europe (Fuhrmann et al., 1983; Lesel et al., 1983; Roberts, 1983).

Because *Y. ruckeri* displays a certain degree of morphological variability (Austin et al., 1982), some confusion exists about its true taxonomic relationships (Green and Austin, 1983). However, isolates of *Y. ruckeri* have highly uniform biochemical (O'Leary et al., 1979) and genotypic (Schill et al., 1984) characteristics. The bacterium was divided initially into two antigenic serotypes correlating with the ability to ferment sorbitol (O'Leary, 1977). Isolates that did not ferment sorbitol and consisted of Serotype 1 strains were commonly associated with frank disease. Alternatively, Serotype 2 fermented sorbitol, was antigenically unrelated to Serotype 1 bacteria, and usually was associated with asymptomatic infections.

Since Serotype 1 bacteria are antigenically homogeneous and pathogenic, immunization has become a practical means for prevention and control of ERM in hatcheries (Bullock and Anderson, 1984). McCarthy and Johnson (1982) demonstrated that vaccination of fish with a Serotype 1 strain of *Y. ruckeri* conveyed a wide degree of cross-protection against other Serotype 1 isolates. Therefore, Serotype 1 bacteria are used exclusively in the preparation of commercial bacterins against ERM disease.

Recently, an epizootic of ERM disease confirmed in chinook salmon (*Oncorhynchus tshawytscha*) was caused by a sorbitol-fermenting Serotype 2 isolate of *Y. ruckeri* (Cipriano et al., 1986). The present study was conducted to determine the relative degree of cross-protection conferred by immunization of fish with bacterins

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prepared with both Serotype 1 and 2 isolates. The specificity and significance of humoral immunity against *Y. ruckeri* was examined also.

MATERIALS AND METHODS

Isolates used in this study were obtained from the reference culture collection maintained at the National Fish Health Research Laboratory (Kearneysville, West Virginia 25430, USA). The two sorbitol nonfermenting Serotype 1 isolates (11.14 and 11.40) and the two sorbitol-fermenting Serotype 2 isolates (11.29 and 11.86) are described elsewhere (Schill et al., 1984; Cipriano et al., 1986).

Bacterins were prepared with isolates 11.29, 11.40, and 11.86 according to the procedure of Amend et al. (1983), with minor modifications. Briefly, cultures were shaken at 150 rpm for 48 hr at 25 C in 1 liter of brain-heart infusion broth (DIFCO, Detroit, Michigan 48232, USA). Cells were pelleted by centrifugation at 3,896 *g* for 30 min at 4 C and resuspended in 5 mM ethylenediamine-tetraacetic acid (EDTA) and 1% glycine. Using buffer as diluent, the suspension was adjusted to 25% transmittance (525 nm) with a Spectronic-20 colorimeter (Bausch & Lomb, Rochester, New York 14625, USA). The suspension was brought to pH 9.5 with 2.5 N NaOH for 60 min and then neutralized by addition of 5 N HCl. After neutralization with HCl, formalin was added to a final concentration of 0.4% and bacterins were checked for sterility by inoculation on brain-heart infusion agar.

Brook trout (*Salvelinus fontinalis*) used for immunization experiments were obtained from the National Fish Hatchery (White Sulphur Springs, West Virginia 24986, USA). These fish were certified to be free of *Y. ruckeri* by procedures recommended by the American Fisheries Society (Amos, 1985). Three groups of 130 brook trout (62.8 ± 1.92 g) each were immunized by intraperitoneal injection with 0.2 ml of bacterin 11.29, 11.40, or 11.86. Fish were held in aquaria receiving a constant flow of 12.5 C spring water. After 4 wk, 10 fish per group were monitored for the presence of serum antibody to homologous and heterologous antigens, using a standard microagglutination procedure (Maisse and Dorson, 1976). Formalin-killed antigens of isolates 11.29, 11.40, and 11.86 were used to monitor agglutinin production. Agglutinin titers used here refer to the reciprocal of the last $\log_2$ dilution of serum yielding a positive agglutination of antigen.

Immediately after antibody analysis the fish, immunized with each bacterin, were randomly subdivided, according to bacterin, into six groups of 20 each. Groups were then challenged with Serotype 1 isolate 11.14 or Serotype 2 isolates 11.29 or 11.86 by exposing them for 1 min in 1 liter of culture containing about 1×10^8 colony forming units (cfu)/ml, as described by Cipriano (1982a). The presence of *Y. ruckeri* was confirmed in kidneys of dead fish by isolation of characteristic colonies on the differential medium described by Waltman and Shotts (1984). Characteristic colonies were further identified by appropriate cytochrome oxidase and triple sugar iron agar reactions. Sorbitol utilization was determined on the medium described by Cipriano and Pyle (1985).

Passive immunization experiments were then conducted to assess the relative significance of humoral antibody and protection against ERM disease. Pools of serum from trout vaccinated with isolates 11.29 and 11.40 were used in these studies. Immune sera and normal serum (0.1 ml/fish) were injected intraperitoneally into groups of 10 each brook trout. After 24 hr, serum antibody levels were monitored in 10 fish per group by the standard microagglutination procedures previously described. Duplicate groups of 10 fish were challenged with either 11.86 or 11.14, 24 hr after injection with immune serum.

RESULTS

Brook trout produced humoral agglutinating antibodies that were highly specific for the particular serotype of *Y. ruckeri* against which they were vaccinated. However, fish vaccinated against either serotype were cross-protected when challenged with homologous and heterologous isolates. Trout immunized 4 wk previously with the Serotype 1 bacterin isolate (11.40) had developed a mean antibody titer of 7.0 ± 0.2 against homologous antigen, but no measurable antibody to either of the Serotype 2 antigens (isolates 11.29 and 11.86). Trout vaccinated with either of the Serotype 2 bacterins (isolates 11.29 and 11.86) did not produce agglutinins against Serotype 1 antigen (isolate 11.40). Similar agglutinin titers were measured in trout to Serotype 2 antigens, regardless of which Serotype 2 bacterin was used for immunization. Trout immunized with the 11.29 bacterin had a mean antibody titer of 6.1 ± 0.31 and 5.5 ± 0.31 against the 11.29 and

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TABLE 1. Mortality in groups of 20 brook trout challenged 4 wk postvaccination with Serotype 1 and 2 bacterins of *Yersinia ruckeri*.

Bacterin strain	Serotype	Challenge strain and group					
		11.29		11.86		11.40	
		1	2	1	2	1	2
11.40	1	0	0	1	0	0	0
11.29	2	0	0	1	0	0	0
11.86	2	0	0	0	0	1	0
Controls		0	0	15	13	6	5

11.86 antigens, respectively. Trout immunized with the 11.86 bacterin had a mean antibody titer of 5.5 ± 0.3 against 11.29 antigen and 5.8 ± 0.3 against 11.86. No agglutinin titers against 11.29, 11.40, or 11.86 antigens were observed in control fish.

Groups of fish were challenged with various concentrations (cfu/ml) of the different isolates: 7.5×10^8 of 11.29, 4.3×10^8 of 11.86, and 8.0×10^8 of 11.14. All fish were protected against experimental challenge, regardless of the bacterin that had been used for immunization (Table 1). No mortality was observed in groups of control trout challenged with isolate 11.29. In other work at the National Fish Health Research Laboratory, isolate 11.29 generally has been avirulent in fish. Inclusion of these data serve as an appropriate control to indicate that there was no mortality among experimental lots of trout caused by physical or nonspecific elements of the immunization regimens and challenge procedures.

TABLE 2. Mean $\log_2$ serum agglutinin titers[a] of brook trout 6 wk postvaccination with bacterins of *Yersinia ruckeri* and 15 days after experimental challenges.

Immunizing strain (serotype)	Challenge strain			Agglutinin antigen
	11.29	11.86	11.40	
11.40 (1)	0.0	0.0	0.0	11.29
	0.0	0.0	0.0	11.86
	8.1 ± 0.2	4.1 ± 0.4	8.0 ± 0.4	11.40
11.29 (2)	5.9 ± 0.3	4.2 ± 0.3	5.9 ± 0.3	11.29
	6.4 ± 0.3	6.8 ± 0.3	7.4 ± 0.4	11.86
	0.0	0.0	0.0	11.40
11.86 (2)	7.8 ± 0.3	6.8 ± 0.2	7.3 ± 0.2	11.29
	8.5 ± 0.4	7.3 ± 0.5	8.8 ± 0.4	11.86
	0.0	0.0	0.0	11.40

[a] Mean ± standard error of 10 fish per group.

TABLE 3. Passive immunization experiments: mortality among groups of 10 brook trout each injected intraperitoneally with either normal (NS) or immune (IS) trout serum after experimental challenge with 1×10^8 bacteria per ml of isolates 11.14 and 11.86.

Challenge strain	Group	Serum injected		
		IS 11.40	IS 11.86	NS
11.14	1	9	8	9
	2	9	8	7
11.86	1	10	9	10
	2	10	10	9

In two groups of 20 control trout challenged with the 11.86 isolate, 15 and 13 fish died. However, not more than one trout died in any of the groups that had been vaccinated with isolates 11.29, 11.86, or 11.40 and challenged with the 11.86 isolate (Table 1). A chi-square analysis (df = 1) indicated that mortality between control and immunized fish differed statistically ($P < 0.01$).

Although the 11.14 isolate was less virulent than 11.86 for brook trout, six and five fish died in the two groups of control fish challenged with 11.14. Again no more than one trout died among groups of immunized trout, regardless of bacterin source. Mortality between control and immunized groups of fish differed statistically (χ^2 analysis; df = 1; $P < 0.05$).

Assays of humoral agglutinating antibody levels in groups of 10 immunized brook trout that survived specific challenge treatment indicated that only serotype-specific antibodies were present (Table 2). Fish immunized with Serotype 2 isolates (11.29 and 11.86) had antibody against these antigens, but not against Serotype 1 strain 11.40. Fish immunized with Serotype 1 bacterin (11.40) had antibody

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against homologous antigen (11.40). Thus, the challenge itself did not evoke the development of cross-serotypic serum antibodies.

Passive immunization studies further indicated that humoral agglutinins were not indicative of the cross-serotypic protection observed in the previously described immunization experiments. The pool of immune serum prepared against the Serotype 1 isolate 11.40 had an antibody titer of 8.9 against homologous antigen but no titer against 11.86. The pool of serum developed against Serotype 2 isolate 11.86 had a titer of 7.5 against homologous antigen and no titer against the 11.40 antigen. Normal serum, used as a control, had no antibody titer against either antigen.

When fish were examined 24 hr after injection with these respective pools of serum, specific antibody was present. Trout injected intraperitoneally with the pool of 11.40 serum had a mean humoral antibody titer of 4.7 ± 0.2 against the homologous antigen but no titer against 11.86. Those injected with the 11.86 serum had a humoral antibody titer of 4.1 ± 0.2 against the homologous antigen but no titer against the 11.40 antigen.

Despite the presence of circulating antibody, mortality did not differ statistically (χ^2 analysis, df = 1; $P > 0.05$) between fish injected with normal and immune sera (Table 3).

DISCUSSION

Vaccination is an effective and economical means for control and prevention of ERM in fishes (Amend and Eshenour, 1980). Such immunization has been directed exclusively against Serotype 1 isolates of *Y. ruckeri* typically associated with diseased fish (McCarthy and Johnson, 1982). O'Leary et al. (1982) suggested that the exclusive use of only Serotype 1 isolates in commercial vaccines might favor increased resistance to Serotype 1 bacteria and also induce the emergence of Serotype 2 isolates as specific causes of ERM disease in fish. Recently, such an isolate was obtained from chinook salmon at a state hatchery in Illinois and the prevalence of reports of the isolation of Serotype 2 bacteria from diseased fish has increased (Cipriano et al., 1986). Consequently, we performed this study to determine if fish vaccinated with Serotype 1 were protected against challenge with Serotype 2 and vice versa.

Serological data presented in this study indicated that brook trout produced highly specific circulating humoral agglutinins only against the specific serotype used for bacterin preparation, but they were protected against experimental challenge with isolates of both serotypes. Analysis of fish that survived 14 days after challenge further indicated that the challenge procedure itself did not evoke the formation of measurable amounts of humoral agglutinins against a serotype other than that used for immunization. These results are consistent with other reports on the specificity of antibody production against *Y. ruckeri* and the lack of a correlation between humoral immunity and the efficacy of vaccination. Anderson et al. (1979) observed that rainbow trout injected with O-antigen preparations from a sorbitol positive Serotype 2 strain of *Y. ruckeri* produced highly specific humoral agglutinating antibodies and plaque forming cells that did not react with antigens from a Serotype 1 isolate. Anderson and Nelson (1974) noted also that rainbow trout orally vaccinated with bacterins of *Y. ruckeri* were protected against disease, but did not develop humoral agglutinins. Cossarini-Dunier (1986) later noted that levels of humoral agglutinins induced by vaccination did not correlate with the degree of protection conferred against experimental challenge.

Our results corroborated the above studies and suggested that protection against *Y. ruckeri* was not dependent on circulating levels of serum agglutinins. This nondependence hypothesis was further

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strengthened by our passive immunization experiments. Consistent with the results of Groberg et al. (1979), we found serotype-specific antibodies in the serum of brook trout within 24 hr after intraperitoneal injection with immune sera. However, these antibodies conferred no protection, since mortality of fish injected with immune sera equaled or exceeded that of fish injected with normal serum. These results are in contrast to reports of successful passive immunization of fishes against other bacterial diseases such as vibriosis (Harrel et al., 1975; Viele et al., 1980) and furunculosis (Spence et al., 1965; Cipriano, 1982b). Therefore, circulating agglutinins are apparently not necessary for the development of practical immunity in fish against *Y. ruckeri*. Other factors, such as a localized or cell-mediated immune responses should be examined to fully understand the mechanism of protective immunity against *Y. ruckeri*.

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