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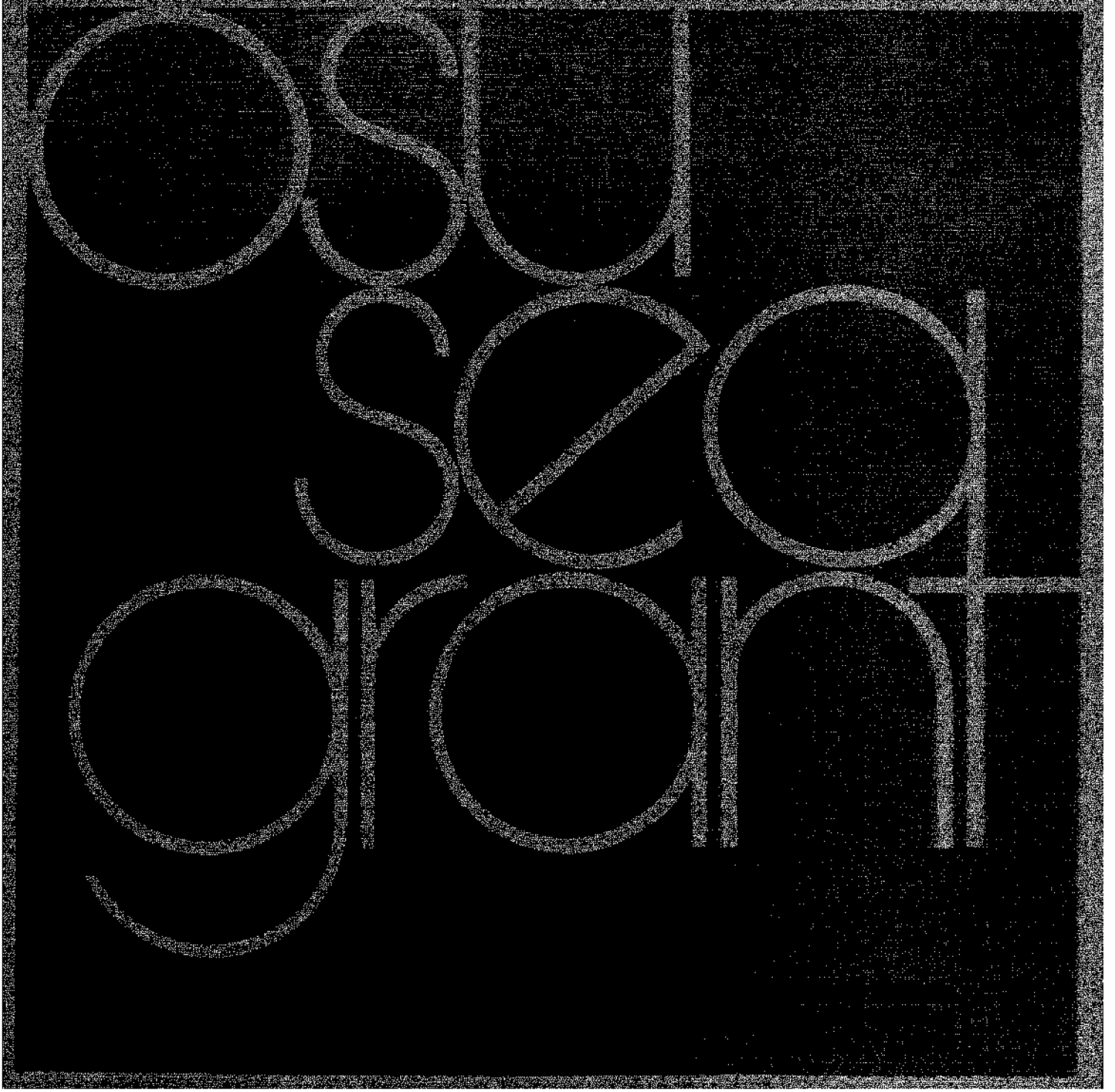

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Technical Paper No. 6235
Oregon Agricultural Experiment Station

Sea Grant College Program
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Publication No.: ORESU-T-82-001

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Acknowledgements

This work is a result of research sponsored by NOAA Office of Sea Grant, Department of Commerce, under Grant No. NA79AA-D-00106 (Project No. R/FSD-5). The U.S. Government is authorized to produce and distribute reprints for governmental purposes notwithstanding any copyright notation that may appear hereon.

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Abstract

Seventeen cultures of *Yersinia ruckeri* isolated from diseased salmonid fish in Oregon and Idaho, U.S.A. and Saskatchewan, Canada, were studied by serological and immunological methods. Based on agglutination reactions, four were found to have an antigenic composition distinctly different from that of the other thirteen. Thus the existence of two serotypes, referred to as I and II, was demonstrated. The group which included the Idaho strain (HI-70) was designated serotype I. Cultures of serotype I also differed from those of serotype II with respect to the fermentation of sorbitol. A bacterin prepared from a formalin killed culture of serotype II given intraperitoneally to juvenile rainbow trout (*Salmo gairdneri*) protected them against infection with cultures of either serotype I or II. Bacterin made from a culture of serotype I, however, provided protection against serotype I infection, but not against serotype II. Bivalent bacterin (serotype I and II) effectively protected fish against infection with either serotype.

Introduction

Enteric redmouth disease, caused by the bacterium *Yersinia ruckeri*, produces both acute and chronic infections of salmonid fish. The disease is of economic importance, especially in certain locations where high density culture of salmonids occurs (Wagner and Perkins 1952, McDaniel 1971, Wobeser 1973, Hester 1973). Historically it has been controlled by improved hatchery sanitation and treatment with antibiotics. Concerns over the emergence of drug resistant strains of bacteria and the limitations placed on the use of new antimicrobial compounds for disease control in fish have caused increased use of bacterins for prophylactic immunization (Ross and Klontz 1965, Anderson and Ross 1972, Anderson and Nelson 1974). Until recently only one serotype of *Y. ruckeri* was known. The work reported here was initiated to determine if one or multiple serotypes were present among populations of diseased fish. This information was considered important for the further development of efficacious bacterins for control of this disease in salmonids.

Materials and Methods

Selection of isolates

Isolates used in this study are listed in Table 1. All conformed to the characteristics designated for *Y. ruckeri* (Ross et al. 1966, Ewing et al. 1978, O'Leary et al. 1979) and were stored in pure culture under sterile mineral oil on Brain Heart Infusion (BHI) agar (Difco).

Preparation of antisera

Isolates HI-70, OS-76, SC-72, BC-74, SP-70, and TH-75 were chosen for serological comparison on the basis of differences in agglutination titer to a known *Y. ruckeri* antiserum and diversity of geographical occurrence. For antigen preparation each isolate was taken from stock cultures, inoculated into BHI broth and incubated for 24 h at room temperature. To insure culture purity, Gram strains were examined with light microscopy and motility determined by means of phase contrast microscopy. Each culture was also grown on BHI agar to observe homogeneity of colony morphology. The cells in broth culture were then killed with formalin (0.3% v/v). After overnight incubation at room temperature, 1 ml of the formalin treated culture was placed in thioglycolate broth to establish sterility.

The killed cells were washed three times with phosphate buffered saline (PBS), pH 7.4, and the packed cells mixed with an equal volume of Freund's complete adjuvant. Approximately 0.7 ml of this preparation was injected intramuscularly into each hind flank of an adult New Zealand white rabbit. Antiserum was harvested after three weeks and stored at -20°C until used.

Slide agglutination tests

One loopful of bacterial cells grown on BHI agar was added to 0.5 ml of PBS and stirred with a vortex mixer to achieve a uniform suspension. To one well of a concave slide one drop each of PBS, bacterial cell suspension, and antiserum was added. To another well two drops of PBS and one drop of bacterial cell suspension were added to serve as a control. The slide was then gently rotated for 2 min. Agglutination of the cells was determined both macroscopically and microscopically with the aid of a dissecting microscope.

Adsorption of rabbit antisera

Each isolate was cultured on BHI agar at room temperature. The cells were then harvested, washed three times in PBS, and used for the adsorption of antisera. Each antiserum was adsorbed with its homologous antigen and with each heterologous isolate by the following procedure: a volume of antiserum was added to an equal volume of packed, washed cells; the cells were resuspended in the antiserum and allowed to incubate for 2 h at 30°C with periodic mixing; the cells were then

Table 1. Cultures of *Yersinia ruckeri* used in this study.

Isolate Code	Location	Date Isolated	Host
BC-74	Big Creek Hatchery, Oregon	1974	Fall Chinook
HI-70	Hagerman, Idaho	1970	Rainbow Trout
SC-72	Saskatachwan Potholes, Canada	1972	Rainbow Trout
SP-70	Salem Pond, Oregon	1970	Chinook Salmon
TH-75	Trask Hatchery, Oregon	1975	Coho Salmon
OS-76	Oak Springs Hatchery, Oregon	1976	Rainbow Trout
RR-70	Roaring River Hatchery, Oregon	1970	Rainbow Trout
SS-75	South Santiam Hatchery, Oregon	1975	Summer Steelhead
SH-75	South Santiam Hatchery, Oregon	1975	Summer Steelhead
ER-75	Elk River Hatchery, Oregon	1975	Fall Chinook
EL-75	Elk River Hatchery, Oregon	1975	Fall Chinook
BC-75	Big Creek Hatchery, Oregon	1975	Fall Chinook
BH-75	Big Creek Hatchery, Oregon	1975	Fall Chinook
WS-75	Willamette Salmon Hatchery, Oregon	1975	Winter Steelhead
WS-74	Willamette Salmon Hatchery, Oregon	1974	Spring Chinook
WT-75	Willamette Trout Hatchery, Oregon	1975	Rainbow Trout
GC-70	Gnat Creek Hatchery, Oregon	1970	Winter Steelhead

removed by centrifugation and the remaining antiserum was adsorbed two additional times by the same method.

Determination of agglutinating antibody titers

The bacterial cell antigens were prepared by growing the appropriate *Y. ruckeri* isolate in BHI broth overnight at room temperature. The cells were washed three times in PBS and resuspended in the same buffer to an optical density of 0.85 at 525 nm. The microtiter system (Cooke Engineering, Alexandria, Virginia) was employed, using 0.25 ml dilutors and pipets. Two-fold dilutions of each serum were made in disposable plastic plates with "U" shaped wells. After the dilutions were made and the bacterial cell antigens added, the plates were gently rotated to provide a uniform cell suspension and sealed with a mylar sheet to prevent evaporation. The plates were incubated for 1 h at room temperature and then overnight at 4°C. The dilution of the last well to show agglutination was the titer of that serum. All titers were determined in duplicate.

Determination of cross protection provided fish by both serotypes of *Yersinia ruckeri*

One millilter of a 12 h culture of isolate BC-74 or HI-70 was inoculated into separate flasks containing 300 ml of BHI broth and incubated at 18°C. After 24 h the cultures were killed by addition of 0.3% formalin (v/v) and allowed to stand for 12 h with periodic mixing. The preparations were examined for sterility by inoculating 1 ml into thioglycolate broth. The cells from each flask were washed three times in PBS and resuspended to an optical density of 1.5 at 525 nm. Rainbow trout (mean weight 28 g) were injected intraperitoneally (i.p.) with 0.1 ml of the appropriate cell suspension. Each group, consisting of 220 fish, was marked with a different color (Scientific Marking Material, Seattle, Washington) by the fluorescent pigment technique (Pribble 1976). The fish were held six weeks at 11°C in fish-pathogen-free well water to allow time for stimulation of the immune response before challenge.

Challenge with the homologous and heterologous isolates was performed after pooling 25 fish from each group into a single aquarium with the water at 12°C. Each group of test fish was recognized by its individual fluorescent pigment mark. The water temperature was raised to 15°C and one LD_{50} of the bacterium contained in 0.1 ml PBS was injected i.p. The experiments were performed in duplicate. Dead fish were collected daily, necropsied, and cultured for the presence of *Y. ruckeri* by streaking kidney tissue on BHI agar.

Results

Serotype determination

Antisera against HI-70, OS-76, SC-72, BC-74, SP-70, and TH-75 were adsorbed with homologous and heterologous cells. The adsorbed and unadsorbed antisera from each isolate were then titered against cells from each of these six isolates (Table 2). Adsorption of HI-70, OS-76, and SC-72 antisera by cells from any one of these strains removed all agglutinating antibody; hence, these isolates were indistinguishable and possessed a common antigen referred to as antigen 1 and were designated serotype I. Adsorptions with BC-74, SP-70, and TH-75 antisera also removed all agglutinating antibody when cells from any of these strains were used. These results indicate the presence of a second major antigen and consitute the evidence for serotype II.

The presence of a third antigen was indicated by agglutination obtained when unadsorbed SC-72 antisera was titered with cells of BC-74, SP-70, and TH-75 (Table 2). Similar levels of antibody were also detected when antisera against BC-74, SP-70, and TH-75 were reacted with SC-72 cells. Reduced titers following adsorption of SC-72 antisera with BC-74, SP-70, and TH-75 cells further indicated the presence of this third antigen. This additional antigen was apparently absent in cells of HI-70 and OS-76 because these cultures failed to react with antisera prepared from BC-74, SP-70, and TH-75 cells.

Slide agglutination reactions with six isolates of *Yersinia ruckeri*

Antiserum prepared against each isolate was tested by slide agglutination with all six cultures of *Y. ruckeri*. Two types of agglutination reactions were observed (Figure 1, c and d). Agglutinated cells with a fine granular appearance, as seen in Fig. 1-d, occurred only when antigen 3 reacted with its homologous antibody. Figure 1-c illustrates a much stronger agglutination reaction which occurred whenever antigens 1 or 2 reacted with their homologous antisera. In this reaction, agglutination appeared more complete, with all cells clumped tightly together (Table 3).

Determination of cross protection between serotypes I and II

The purpose of this experiment was twofold: (1) to determine if bacterins prepared from the two serotypes of *Y. ruckeri* could provide cross protection against infection in vaccinated fish and (2) if such bacterins are not cross protective, to determine whether a bivalent bacterin consisting of both serotypes would give such protection. Isolates HI-70 and BC-74 were chosen to represent serotypes I and II, respectively, and isolate SC-72 was included to determine the significance, if any, of antigen 3. Juvenile rainbow trout were vaccinated and divided into paired lots of 25 fish each. Six weeks later they were challenged by i.p. injection with one LD_{50} of a culture of either HI-70, BC-74, or SC-72 (Table 4). Unfortunately, SC-72 failed to provide an adequate level of challenge.

Table 2. Serological comparison of *Yersinia ruckeri* isolates HI-70, OS-76, SC-72, BC-74, SP-70 and TH-75.

Antisera	Adsorbed with antigen	Agglutination titer when tested with *Y. ruckeri* antigen						Antigens in corresponding organism
		HI-70	OS-76	SC-72	BC-74	SP-70	TH-75	
HI-70	Unadsorbed	256	512	512	0	0	0	1
	HI-70	0	0	0	0	0	0	
	OS-76	0	0	0	0	0	0	
	SC-72	0	0	0	0	0	0	
	BC-74	256	256	256	0	0	0	
	SP-70	128	256	128	0	0	0	
	TH-75	256	256	64	0	0	0	
OS-76	Unadsorbed	1024	2048	2048	0	0	0	1
	HI-70	0	0	0	0	0	0	
	OS-76	0	0	0	0	0	0	
	SC-72	0	0	0	0	0	0	
	BC-74	512	2048	1024	0	0	0	
	SP-70	512	1024	512	0	0	0	
	TH-75	512	512	1024	0	0	0	
SC-72	Unadsorbed	128	128	2048	4	4	8	1,3
	HI-70	0	0	0	0	0	0	
	OS-76	0	0	0	0	0	0	
	SC-72	0	0	0	0	0	0	
	BC-74	64	128	128	0	0	0	
	SP-70	64	128	64	0	0	0	
	TH-75	64	128	128	0	0	0	
BC-74	Unadsorbed	0	0	4	1024	512	1024	2,3
	HI-70	0	0	0	512	512	256	
	OS-76	0	0	0	512	512	512	
	SC-72	0	0	0	512	256	256	
	BC-74	0	0	0	0	0	0	
	SP-70	0	0	0	0	0	0	
	TH-75	0	0	0	0	0	0	
SP-70	Unadsorbed	0	0	8	2048	256	512	2,3
	HI-70	0	0	0	1024	256	256	
	OS-76	0	0	0	2048	256	256	
	SC-72	0	0	0	1024	128	256	
	BC-74	0	0	0	0	0	0	
	SP-70	0	0	0	0	0	0	
	TH-75	0	0	0	0	0	0	
TH-75	Unadsorbed	0	0	4	2048	1024	512	2,3
	HI-70	0	0	0	512	256	256	
	OS-76	0	0	0	512	256	512	
	SC-72	0	0	0	512	128	512	
	BC-74	0	0	0	0	0	0	
	SP-70	0	0	0	0	0	0	
	TH-75	0	0	0	0	0	0	

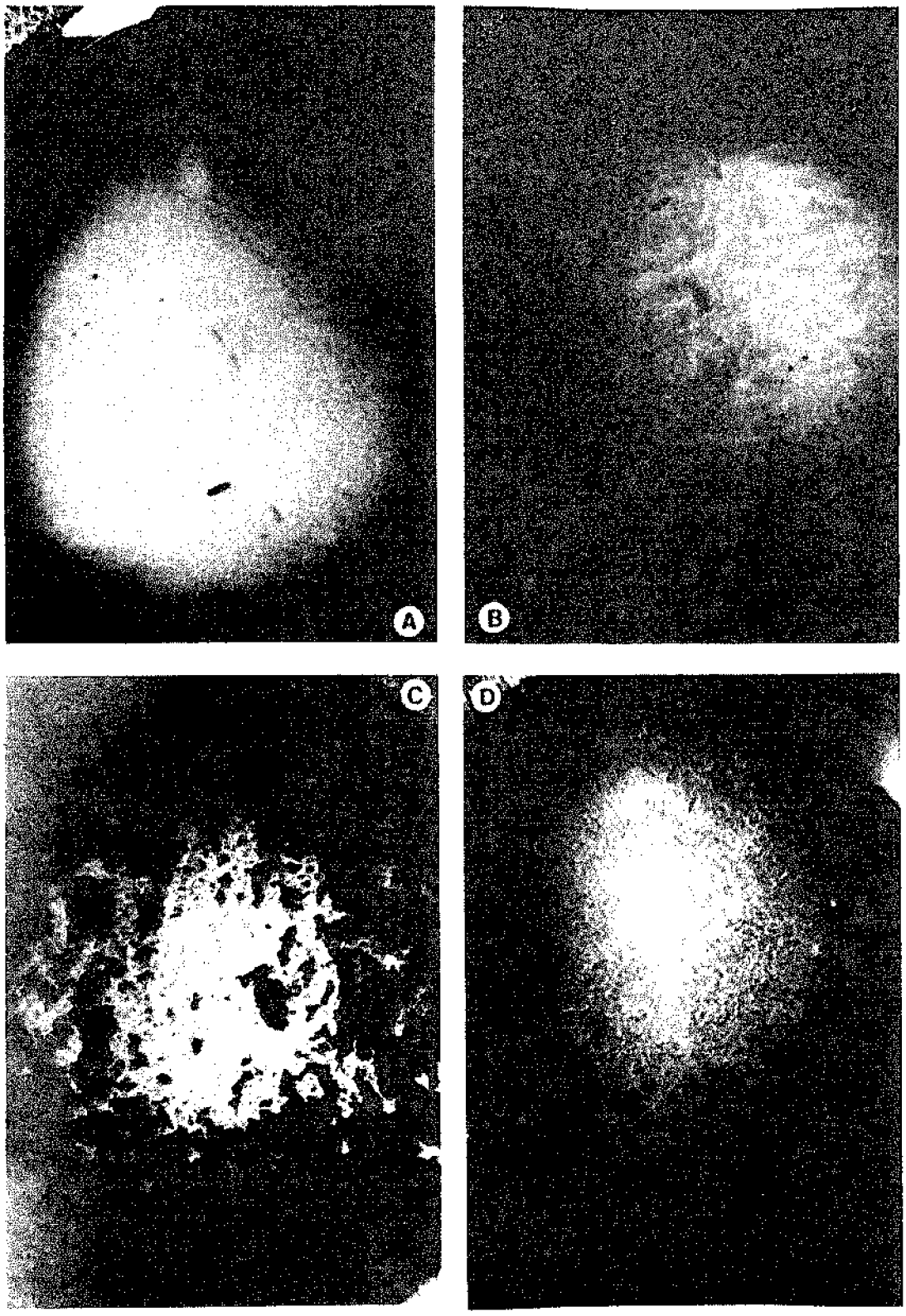

Fig. 1. Slide agglutination tests with *Yersinia ruckeri*: (a) PBS control (b) normal rabbit serum control (c) positive agglutination of either antigens 1 or 2 and (d) positive agglutination of antigen 3.

Table 3. Results of slide agglutination tests among six isolates of *Yersinia ruckeri*.

Cells agglutinated against	Antisera against isolate: HI-70	OS-76	SC-72	BC-74	SP-70	TH-75	Cell Antigens	Serotype
HI-70	+	+	+	-	-	-	1	I
OS-76	+	+	+	-	-	-	1	I
SC-72	+	+	+	±	±	±	1,3	I
BC-74	-	-	±	+	+	+	2,3	II
SP-70	-	-	±	+	+	+	2,3	II
TH-75	-	-	±	+	+	+	2,3	II

+ = the agglutination reaction shown in Figure 1c.

± = the agglutination reaction shown in Figure 1d.

- = the agglutination reaction shown in Figure 1a or 1b.

Table 4. Comparison of protection of juvenile rainbow trout (*Salmo gairdneri*) against *Yersinia ruckeri* by injection of monovalent and bivalent bacterins.

Isolate bacterin prepared from	Isolate used for challenge[a]		Combined percent mortality[b]	
	BC-74[c]	HI-70[c]	BC-74	HI-70
BC-74	7/25 2/25	7/25 0/25	18[d]	14[d]
HI-70	20/25 18/25	3/25 0/25	76	6[d]
SC-72	7/25 2/25	2/25 1/25	18[d]	6[d]
BC-74 combined with HI-70	6/25 1/25	2/25 1/25	14[d]	6[d]
unvaccinated controls	21/25 17/25	22/25 21/25	76	86

[a] Fish were injected with one LD_{50} of BC-74 (5.95×10^6 cells) or one LD_{50} of HI-70 (4×10^6 cells).

[b] *Yersinia ruckeri* was isolated from all mortalities.

[c] Numbers indicate fraction of challenge group that died. Experiments performed in duplicate.

[d] These values were significantly different from those obtained with control groups (Snedecor and Cochran, 1973).

The data indicate that the BC-74 bacterin (serotype II) gave significant protection against challenge with HI-70 (serotype I), but the HI-70 bacterin did not protect against challenge with BC-74. Isolate SC-72 had been shown to possess antigens 1 and 3. Antigens 2 and 3 were found on isolate BC-74 while isolate HI-70 had only antigen 1. The only antigen in common between BC-74 and SC-72 is antigen 3. Fish immunized against either of these cultures and challenged with BC-74 reacted similarly (18% mortality) suggesting that antigen 3 is also protective.

Isolates HI-70 and BC-74 have no agglutinating antigens in common. Fish receiving the HI-70 bacterin showed significant protection when challenged with the HI-70 culture, but were not protected against challenge with BC-74. Conversely, even though no antigens were in common between BC-74 and HI-70, fish vaccinated against either of these isolates were protected when challenged by HI-70. These results may suggest the presence of an alternate type of enhanced resistance to this pathogen. Fish that received the bivalent bacterin composed of cells of both BC-74 and HI-70 were protected when challenged by cultures of either isolate.

Discussion

Serological reactions are extensively used for diagnosis and detection of the causative agent of enteric redmouth disease, *Y. ruckeri*. Therefore, the serological analysis reported here attempts to duplicate that used in most diagnostic laboratories. Analysis demonstrated that at least three distinct antigens could be detected among the six *Y. ruckeri* isolates chosen for comparison. Slide agglutination reactions involving these antigens identified as belonging to either serotype I or II were more complete and macroscopically visible, while agglutination with the third antigen was less complete and had a granular appearance (Figure 1, c and d). On the basis of isolation precedence and the more common occurrence, we have placed isolates similar to HI-70 into serotype I, and those strains similar to BC-74 have been designated serotype II. This is in concurrence with information recently published by Stevenson and Daly (1982). No designation for an additional serotype is made by us because further work is required to determine the importance of the third antigen.

Reports have indicated *Y. ruckeri* cultures either fail to ferment sorbitol (Ross et al. 1966, Busch 1973, Bullock and Snieszko 1975) or ferment it weakly (Ewing et al. 1978, Stevenson and Daly, 1982). We have reported that four of the 17 cultures listed in Table 1 of this report ferment sorbitol (O'Leary et al. 1979). The four strains of *Y. ruckeri* serologically identified as type II (BC-74, BC-75, SP-70, and TH-75) were sorbitol positive, whereas the 13 serotype I isolates were all sorbitol negative. The significance of this deserves further evaluation and will require isolation and testing of additional strains of this bacterium.

Formalin-killed bacterin prepared from a culture of serotype II and administered i.p. to juvenile rainbow trout immunized them against infection with cultures of either serotype; however, a bacterin prepared from a serotype I culture only provided protection against challenge with that serotype. At present bacterins for control of enteric redmouth disease in salmonids only contain serotype I. It appears that serotype I is the most common form of *Y. ruckeri* associated with epizootics in fish and may suggest a greater virulence; however, serotype II has been isolated not only from the Pacific salmon (genus *Oncorhynchus*) but also from diseased rainbow and cutthroat (*Salmo clarki*) trout. Based on performance of the current product(s) we are not aware of any immediate need for the addition of serotype II to commercially prepared bacterins (McCarthy and Johnson 1982). However, continued use of only serotype I may select for and favor the emergence of serotype II among salmonid populations due to the increased level of resistance to serotype I as a direct result of immunization. We observed the increased prevelance of *Vibrio anguillarum* serotype II (*Vibrio ordalii*) only after repeated immunization of large numbers of salmonids with *V. anguillarum* serotype I bacterin. Therefore it is possible that modification (to include serotype II) of the bacterin currently used for control of enteric redmouth disease may be required in the future.

Acknowledgements

This research was supported by the Oregon Department of Fish and Wildlife under PL-89304 Anadromous Fish Act and the Oregon State University Sea Grant College Program supported by NOAA Office of Sea Grant, Department of Commerce under Grant No. NA 79 AA-D-00106. The authors wish to thank Dr. K. S. Pilcher for his contribution in the preparation of this manuscript.

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