ABSTRACT

JENKINS II, THOMAS BURTON. Efficacy of Immersion and Intraperitoneal Vaccination Against *Yersinia ruckeri* and the Effects on Immune Response in Rainbow Trout, *Oncorhynchus mykiss* (Under the direction of Dr. Jeffrey M. Hinshaw).

In this study, the effectiveness of vaccination against *Yersinia ruckeri*, using an autogenous bacterin, in rainbow trout (*Oncorhynchus mykiss*) was assessed. In addition, the immune response detected by enzyme-linked immunosorbent assay (ELISA) was evaluated. Rainbow trout were vaccinated at 25 grams mean body weight by standard bath immersion and intraperitoneal (i.p.) injection (saline-based injection or oil-based adjuvant injection). Bath-challenges occurred on days 42, 101, 181, 286, and 381 post-vaccination. Plasma samples were collected pre-challenge and fourteen days post-challenge for antibody titer analysis. Antibody kinetics were followed prior to first challenge. Relative percent survival (RPS) ranged from 98.96% to 96.85% and did not statistically differ among vaccinated groups. Increased antibody titer was identified in the oil-based adjuvant injection vaccinated group however, statistical significance among vaccinated groups was not determined.

EFFICACY OF IMMERSION AND INTRAPERITONEAL VACCINATION AGAINST *YERSINIA RUCKERI* AND THE EFFECTS ON IMMUNE RESPONSE IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

by

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BIOGRAPHY

Thomas Burton Jenkins II (Burt) was born in Gastonia, North Carolina on February 6, 1972 and moved to Burlington, Kansas in 1980. Burt graduated from Burlington High School in 1990 as class valedictorian and then enrolled at Independence Community College to pursue his academic and athletic goals. The author earned his Associate of Science degree in Biology in 1992. Burt then moved to Asheville, North Carolina to further his academic and athletic pursuits. While at the University of North Carolina-Asheville Burt played varsity men's basketball and in 1995 earned a Bachelor of Arts degree in Biology.

Upon completion of his undergraduate degree, Burt removed himself from the academic arena for a year. During this time, Burt became the manager of the fishing department at a Raleigh sporting goods store. After a year of working, Burt entered North Carolina State University to pursue further academic goals. Burt entered the Fisheries and Wildlife Science program under the direction of Dr. Jeffrey M. Hinshaw. Academic work was completed at the university campus and project specific work was done at the Mountain Horticultural Crops Research and Extension Center and the North Carolina State University College of Veterinary Medicine. This led to the culmination of the work presented here.

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TABLE OF CONTENTS

Page

LIST OF APPENDICES

LIST OF TABLES

LIST OF FIGURES

1. *Yersinia ruckeri*, the Causative Agent of Enteric Redmouth Disease

1.1 Introduction

Yersinia ruckeri, the causative agent of Enteric Redmouth Disease (ERM), has been extensively studied since its first discovery in the 1950's. ERM was the first fish disease for which a commercially available bacterin was developed (Bullock 1984). ERM is currently found in the U.S., Canada, England, Europe, and Australia (Wobeser 1973; Bentley 1982; Fuhrmann et al. 1983; Lesel et al. 1983; Giorgetti et al. 1985).

1.2 Serological Variations of the Bacterium

Yersinia ruckeri was first isolated from rainbow trout by R.R. Rucker in the 1950's at Hagerman, Idaho (Ross et al. 1966). Ross et al. (1966) first described the bacterium as a rod-shaped, gram-negative, catalase-positive, oxidase-negative bacterium isolated from the kidneys of infected rainbow trout. Later, Ewing et al. (1978) confirmed that the bacterium was a member of the family *Enterobacteriaceae*. Studies of serological variation among strains, on the basis of whole cell serology, has resulted in the designation of six different serovars: Serovar I (Hagerman), serovar I' (SBS), serovar II (Oregon and O'Leary), serovar III (Australian), serovar V (Colorado), and serovar VI (Ontario) (Ross et al. 1966; Bullock et al. 1978; O'Leary et al. 1979; Bullock and Anderson 1984; Stevenson and Airdrie 1984; Daly et al. 1986; DeGrandis et al. 1988). New studies based on O-serotyping and lipopolysaccharide (LPS) patterns have described

additional serotypes not mentioned above (Pyle and Schill1985; Davies 1990; Romalde et al. 1993).

1.3 Isolation and Identification

Bacterial isolation is done by aseptic sampling of the kidney, spleen, liver, heart, and lower intestine of fish (Furones et al. 1993). *Y. ruckeri* grows well on general-purpose media such as trypticase soy agar (TSA). Colonies are 1-2mm in diameter, smooth, round with entire edges, and white cream in color (Ross et al. 1966). Identification of the bacterium is determined by biochemical characteristics, summarized in Table 1.3.1.

Table 1.3.1. - Biochemical characteristics of *Y. ruckeri.*

Test	Result	Test	Result
Gram	negative	Methyl red	positive
Shape	rod	Voges Proskauer	variable
Motility	variable	Citrate utilization	positive
G+C ratio	47.5-48.5%	Gelatin hydrolysis	variable
Catalase	positive	Tween 80	variable
Oxidase	negative	Urease	negative
O/F reaction	fermentative	Acid from	
β-galactosidase	positive	arabinose	negative
Arginine dihydrolase	negative	fructose	positive
Lysine decarboxylase	positive	glucose	positive
Ornithine decarboxylase	positive	lactose	negative
Indole	negative	maltose	positive
Hydrogen sulphide	negative	ribose	positive
Tryptophan deaminase	negative	sorbitol	variable
Casein hydrolysis	variable	sucrose	negative
Nitrate reduction	positive	trehalose	positive

(from Furones et al. 1993)

1.4 Characteristics of Infection

ERM mainly affects salmonids but *Y. ruckeri* bacteria has been isolated from crayfish, birds, mammals, and many other fish species (Furones 1993). Infection in salmonids usually occurs in spring and early summer when water temperatures rise (Busch 1978). In the early stages of infection, fish appear dark in color and are very lethargic. Characteristic reddening of the mouth, tongue, jaw area, and operculum may be present. Subcutaneous hemorrhaging at the base of the fins, rays, ventral surfaces, and at the anus may also occur (Rucker 1966; Bullock et al. 1976; Busch 1978). Internal symptoms include petechial hemorrhaging of the liver, pyloric cecae, adipose tissue, pancreas, swim bladder, gonadal tissue, and body musculature. The spleen is usually enlarged and the intestine may become filled with an opaque, yellow, mucoid or watery substance with the intestines themselves becoming flaccid and translucent (Rucker 1966; Busch1978; Bullock 1984). However, characteristic symptoms are not always seen in outbreaks of ERM (Bentley 1982; Roberts 1983; Austin et al. 1986). Infection in larger fish is usually chronic and less severe than in smaller fish. Busch and Lingg (1975) determined that up to 25% of fish surviving infection had become carriers of the disease, resulting in cyclical outbreaks and mortalities by intestinal shedding of *Y. ruckeri.*

Transmission of the bacteria is horizontal through the water (Busch 1978) and reports of horizontal spread have come from both hatchery and laboratory populations (Ross et al. 1966; Rucker 1966; Bullock et al. 1976; Hunter et al. 1980; Bullock 1984). *Y. ruckeri* also has the ability to survive starvation in fresh and marine waters and sediments for more than three months. Romalde et al. (1994) demonstrated that *Y.*

ruckeri can become dormant under certain environmental conditions and can be recultured with no loss of metabolic activity.

1.5 Aspects of Infection

ERM disease is most severe at water temperatures of 15-18°C with mortalities less severe over 20°C and below 10°C (Ellis 1988, Rucker 1966). Handling stress may induce outbreaks of ERM when water temperatures exceed 13°C (Busch and Lingg 1975). Incubation time from initial exposure until first mortality is approximately 4-8 days at 15°C (Busch 1978). Outbreaks may last several months causing sustained, low-level mortalities that result from stresses due to handling, abnormal dissolved oxygen levels, over-crowding, fluctuating water temperatures, and elevated levels of suspended solids, ammonia, or other metabolites (Busch 1978; Bentley 1982; Bullock 1984; Austin et al. 1986; Meir 1986, Caldwell and Hinshaw 1995).

1.6 Vaccination Strategies

Vaccination techniques to control ERM include injection, immersion, bath, spray, oral, and anal intubation. Injection vaccination confers the greatest protection and oral vaccination confers the least protection (Ellis 1988). Good husbandry practices and immunization may help prevent outbreaks of ERM. Vaccination can lower economic losses from mortalities, improve feed conversion, and reduce length of time to marketability (Amend and Eshenour 1980; Tebbit et al. 1981; Horne and Robertson 1987, Lillehaug 1989).

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2. Kinetics and Detection of the Antibody Response After Bath Challenge

2.1 Introduction

Several structures are important in the development of a mature immunologic response in fish. The teleost kidney is an important lymphoid organ, considered to be a source of hemopoietic stem cells. Antigen-presenting cells, and T-like and B-like lymphocytes have been found in the kidney (Iwama and Nakanishi 1996). The thymus is regarded as a primary lymphoid organ where naïve lymphocytes are produced and lymphocyte differentiation and T cell development and education occur. The spleen contains blood in sinuses and ellipsoids (specialized capillary walls composed of reticulin fibers and macrophages), and these areas are believed to function in trapping immune complexes for the development of immune memory (Ellis 1980; Rombout 1993; Evans 1998).

Although all mechanisms of the fish immune response have not been clearly determined, evidence suggests that antibody production in teleosts is similar to that found in mammals (Vallejo et al. 1992). Antigen presenting cells (APC's), namely macrophages, dendritic cells or B-lymphocytes capture antigens. Antigens are then processed biochemically to expose epitopes contained in the internal configuration of the antigen. These epitopes are then expressed on the surface of the APC by the major histocompatibility complex (MHC). The MHC Class I or MHC Class II bound fragments are then recognized by the clonotypic α/β T cell receptor of either $CD4^+$ or $CD8^+$ cells

inducing activation, clonal proliferation, and antibody secretion (Vallejo et al. 1992; Paul 1993; Evans 1998; Janeway et al. 1999)

The capability of immunological memory in fish has been demonstrated in immunization against many pathogens (Johnson et al. 1982). Although fish possess immunological memory, secondary response to a hapten carrier antigen resulted in no increase in affinity maturation (Arkoosh and Kaattari 1991; Evans 1998). The absence of affinity maturation is in contrast to that found in mammals where maturation does occur in response to a secondary exposure, where B cells elicit increased proliferation, and where isotype switching from IgM to IgG occurs along with somatic hypermutation.

Enzyme-linked immunosorbent assays (ELISA) have been developed for the detection of many fish pathogens. ELISA procedures were first reported in 1971 (Engvall and Parlmann 1971; Van weeman and Schurrs 1971) and since have been used to demonstrate antibody activity in fish serum (Roberson 1981; Smith 1981; McArthur and Segupta 1982; Bortz et al. 1984; Chart et al 1984; Hamilton et al. 1986; Neuman and Trip 1986; Thuvander et al. 1987). Cossarini-Dunier (1985) developed an ELISA to detect and quantify specific antibodies in rainbow trout to *Y. ruckeri*.

The purpose of this study was to evaluate the kinetics of the antibody response and to evaluate differences in antibody response, after vaccination, in rainbow trout, *Oncorhynchus mykiss*, vaccinated against *Yersinia ruckeri* by immersion and intraperitoneal injection (saline-based injection and oil-based adjuvant injection) following bath challenge. Circulating antibody levels resulting from the autogenous North Carolina bacterin were measured by ELISA.

2.2 Materials and Methods

Maintenance of Fish

Rainbow trout with no history of exposure to ERM were obtained from Tellico Trout Farm, Franklin, North Carolina, at approximately 500-fish/ kg. Fish were maintained in a recirculating fresh water system (13.5°C-18.0°C) and fed commercial trout feed of appropriate size for the fish.

Vaccination

Johnson et al. (1982a,b) found immunity in salmonids increased with size (range 1-4g) suggesting that even though immunity is detected in smaller fish, immunological memory may take longer to develop. Thorburn and Jansson (1988) found 6.3g fish had lower mortalities than 4.1g fish exposed to *Vibrio anguillarum* after vaccination, further supporting maturation of immune response with fish size. Tebbit et al. (1981) found vaccination above 15 grams allows for development of immunity and reduces vaccination expense by excluding early mortalities due to other causes. Autogenous ERM vaccine (Alpharma NW Inc., Bellevue, Washington) was administered by immersion and intraperitoneal injection following recommended dosages to fish with an average weight of 25 grams. For immersion vaccination, vaccine was diluted 1:10 in clean water. Fish were immersed for 20 seconds at <0.5 kg/L. For injection vaccination, fish were anesthetized in 75L of water from the holding tank with a mixture of 379g (0.5%) NaCl, 11.3g (100 ppm) $NaHCO_3$, and 7.6g (100 ppm) tricaine-methane-sulfonate (MS-222). Fish were injected (0.1 ml/fish) with either a saline-based or an oil-based adjuvant enhanced autogenous vaccine. A control group was established with no vaccination

administered. After vaccination, fish were held in separate cages relative to their vaccine method.

Bacterial Cultures

All bacterial cultures used in this study were reconstituted from the same isolate used to produce the autogenous vaccine. *Y. ruckeri* bacteria were reconstituted from frozen stock in 100 ml sterile Tryptic Soy Broth (TSB) and incubated 24 hours at 22°C, with gentle stirring. Aliquots (10 ml) of the 24 hour culture were placed in each of 8 Erlenmeyer flasks containing 1 L sterile TSB and incubated 24 hours at 22°C, with gentle stirring. Cultures were then pooled and bacterial densities read by spectrophotometer at 525 nm and checked by serial dilution run in triplicate on TSA plates incubated 48 hours at 22°C. Plate counts were used to estimate the culture bacterial density by the following:

$$\text{Density} = \frac{N}{V \times D}$$

Where N = mean colony forming units (cfu) at optimum dilution

V = volume of optimum dilution plated

D = dilution factor

Production of Control Positive and Control Negative Fish Plasma for ELISA Tests

Fish for the production of control positive plasma were produced at the Pisgah Fish Hatchery (Pisgah Forest, North Carolina). Six rainbow trout (approximately 0.75kg.) were injected (0.3ml) with the *Y. ruckeri* vaccine (Alpharma NW Inc., Bellevue,

Washington) containing oil adjuvant. On day 30, fish were caudally bled and the samples were put on ice for further processing. In the laboratory, blood samples were centrifuged for 10 minutes and the plasma was removed with sterile pipettes and placed in screw-cap cryogenic vials labeled and stored at -80°C.

Negative control plasma was obtained from rainbow trout produced at the Erwin National Fish Hatchery (Erwin, Tennessee). This facility is tested semi-annually for specific pathogen free (SPF) certification for reportable diseases. The animals used to obtain the negative control plasma were expected to be *Y. ruckeri* free, non-immunized animals with little chance of prior exposure to *Y. ruckeri*. Blood samples were removed from 4 fish and processed, as previously described for the control positive fish, labeled, and stored at -80°C.

Challenge

Before challenges began lethal concentration (LC_{50}) trials were conducted to establish exposure to proper bacterial densities. Exposure levels during the challenges and LC_{50} trials were determined by:

$$X = \frac{C1 \times V}{C2}$$

Where X = volume of inoculum required (ml)

C1 = desired concentration of pathogen for exposure (cfu/ml)

V = bath water volume (ml)

C2 = concentration of inoculum (cfu/ml)

LC_{50} trials resulted in a target dose of approximately 1.0×10^7 cfu/ml to achieve approximately 50% mortality in the control groups (data not shown). Actual challenge

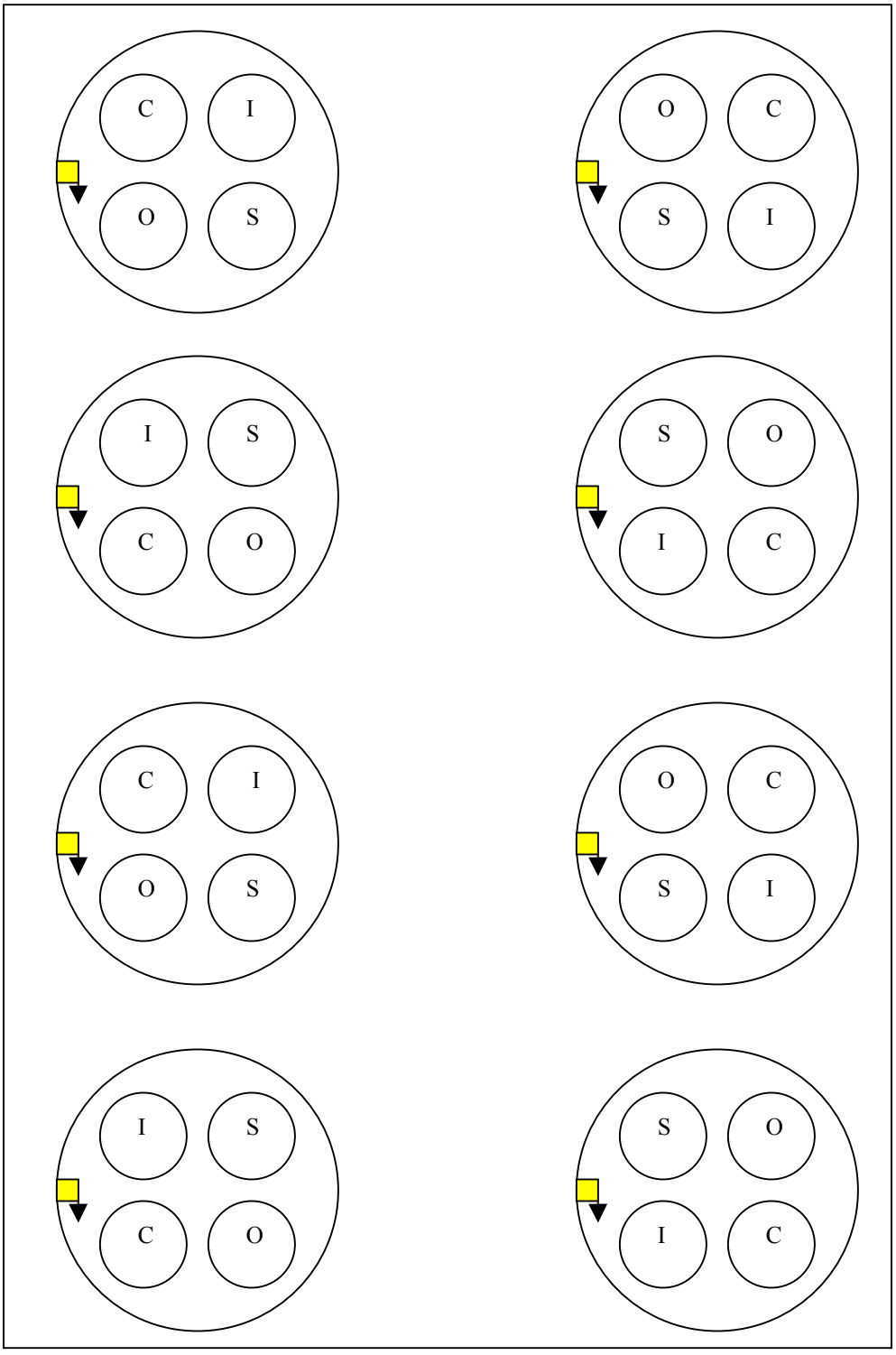
doses were $2.36x10^7$, $1.77x10^7$, $1.79x10^7$, $1.82x10^7$, and $2.01x10^7$ cfu/ml. Water temperatures at challenge were 10.5°C, 14.5°C, 16.5°C, 17.9°C and 13.6°C. Challenge temperatures did not differ from rearing tank temperatures by more than $\pm$ 2°C.

Prior to each challenge 20 fish were sampled, as described previously, for detection of pre-exposure antibody levels. Fish were challenged on days 42, 101, 181, 286, and 381 post-vaccination by bath immersion. Fish were removed from their holding cages and placed in separate 68L tanks containing 19L water. Next, 200 ml of the *Y. ruckeri* broth, prepared as previously described, was introduced to the tanks to give the proper test bacterial concentration as determined from the LC_{50} tests. After a 30-minute challenge fish were placed into their appropriate cages and monitored for 14 days post-exposure. Eight replicates (tanks) were evaluated with each of the 4 test groups contained in each replicate (Figure 2.1). Cages were rotated one position, in reference to inflow, in each tank with each position being replicated twice for analysis of positional effects within tanks. Fish were not fed during challenge.

At 14 days post-exposure 10 surviving fish from each cage (in each tank) were randomly sampled and bled, processed and stored for ELISA analysis.

Figure 2.1. - Tank diagram showing position of vaccinated groups during the challenges.

- Inflow, C- Control (non-vaccinated), O- Oil-based adjuvant injection vaccinated, S- Saline-based injection vaccinated, I- Immersion vaccinated.



Collection of Plasma Samples

Fish were removed from their respective groups and anesthetized as described above. Samples were collected once prior to vaccination (day 0) then on days 2, 4, 6, 8, 10, 12, 14, 21, 28, and 35 post-vaccination. Samples were also collected on days 42, 101, 181, 286, and 381 prior to bacterial challenge. Twenty fish from each test group were sampled on each sampling date. Approximately 0.3 ml. of whole blood was collected via a 21g X 3.8 cm. needle into 3 ml. syringes, previously coated with a solution containing 5 IU of sodium heparin, from the caudal vein. Blood was transferred to 1.5 ml micro centrifuge tubes and kept on ice prior to processing. Any fish with evidence of prior sampling were returned without being bled.

In the laboratory, blood samples were centrifuged for 10 minutes and the plasma was removed with sterile pipettes and placed in screw-cap cryogenic vials, labeled with date, group, and number. All samples were stored at -80°C.

Production of Lipopolysaccharide (LPS) for ELISA Tests

LPS, an endotoxin derived from the cell walls of gram-negative bacteria (Jann and Westphal 1975), was prepared using an isolate of *Yersinia ruckeri* from an outbreak in North Carolina (Appendix 2.1). This isolate shows both Type I and Type II characteristics from LPS comparison to both Type I and Type II serotypes and is resistant to Tetracycline® and Romet® (Wenzel 1996).

Purification of Immunoglobulin (Ig), Production of Rabbit Anti-trout Serum, and ELISA

The methods of Cobb et al. (1998) were followed for affinity purification of rainbow trout Ig (Appendix 2.2) and also for the production of rabbit anti-trout Ig antibodies (Appendix 2.3). An ELISA procedure, developed at the North Carolina State University College of Veterinary Medicine, was followed to measure the amount of antibody activity (antibody titer) against *Y. ruckeri* (Appendix 2.4).

Statistical Analysis

The plasma blood samples drawn at days 2, 4, 6, 8, 10, 12, 14, 21, 28, 35, 42, 101, 181, 286, and 381 were used for analyzing antibody kinetics. The plasma blood samples drawn at days 42, 101, 181, 286, and 381 were used for analyzing post challenge antibody response. For antibody kinetics and post challenge antibody response, the analysis was performed for each day. Both analyses were performed using an ANOVA model with SAS General Linear Models Procedure. A one-factor ANOVA model with treatment was used to analyze antibody kinetics. A three-factor ANOVA model with treatment, tank, and position was used to analyze post challenge antibody response. Statistical significance was determined at an α level of ≤ 0.05. For purposes of analysis, ELISA values (EV) less than 0 were set equal to 0 (measured antibody responses less than the negative control value). Since assumptions for the ANOVA model were not met for the raw ELISA values (normal distribution and constant variance), a log transformation of the raw values was used. For each analysis, 1 was added to each raw

ELISA value. By using log transformation, inequalities in variance among treatment groups were reduced (Steel et al.1997).

The null hypothesis was that there was no difference due to vaccination method. The alternative hypothesis was that there was a significant difference resulting from vaccination method. More specifically, the analyses were to test whether vaccinated groups would produce higher antibody titers (EV) than the non-vaccinated group with the injection-vaccinated groups producing a higher titer than the immersion-vaccinated group. Also, the oil-based adjuvant injection group would produce a higher titer than the saline-based injection vaccinated group. If the F-test for the overall model at each day was significant, individual comparisons among treatment groups were performed.

2.3 Results

A summary of the ANOVA statistics for kinetics is shown in Table 2.1. Statistically significant differences for the overall model were found on all days after day 14. Although the overall model showed significant differences in treatment groups for days 4, 6, and 10, the individual treatment group analyses showed no consistent results (Figure 2.2). Individual treatment group analyses for day 21 through day 286 showed the oil adjuvant injected group was significantly higher than all other groups with the exceptions of the control group on days 21 and 286 and the immersion vaccinated group on day 101. However, at day 381 this significance was not found. Generally, no differences were seen in antibody titer (mean logEV) between the saline-based injected group, the immersion-vaccinated group, and the non-vaccinated (control) group. These

groups were frequently not significantly different from the non-vaccinated group and when significance did occur the relationships (titer higher or lower) were not consistent.

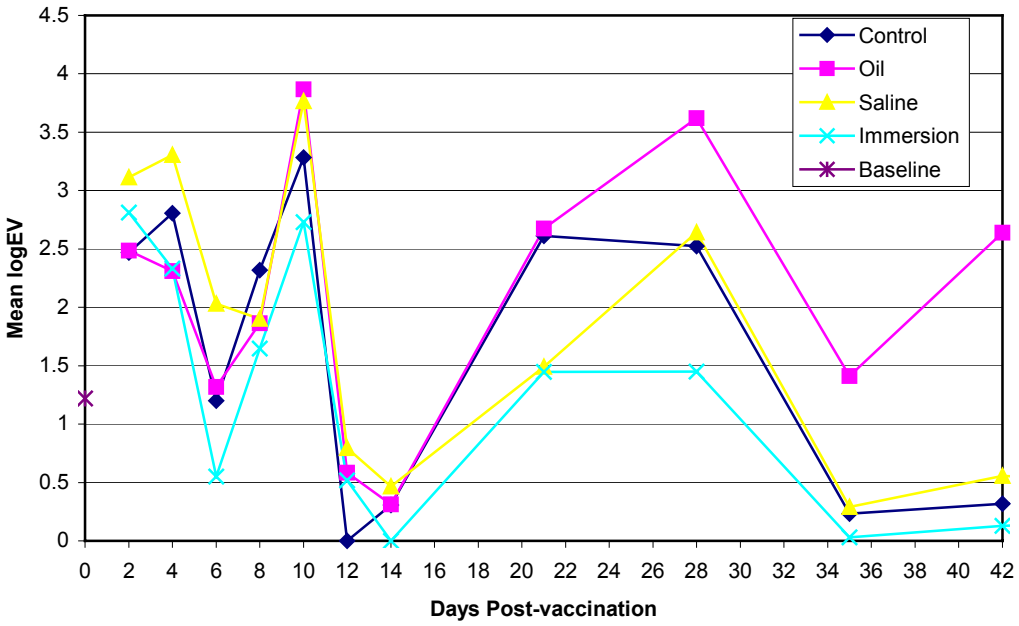
Table 2.1. - Two tailed ANOVA statistics for kinetics of the serum antibody response of rainbow trout following vaccination by various delivery methods. Significance is expressed at $\alpha \leq 0.05$. Control (C), Oil (O) - oil adjuvant injected group, Saline (S) – saline-based injected group, Immersion (I) – standard immersion vaccinated group.

Day	F test (Model)	Method	logEV (avg)	t test (comparisons) C vs. O	C vs. S	C vs. I	O vs. S	O vs. I	S vs. I
0			1.2205						
2	0.1647	Control	2.4706						
		Oil	2.4859						
		Saline	3.1136						
		Immersion	2.8127						
4	0.003	Control	2.8051	0.0989	0.0947	0.1137	0.0012	0.9441	0.0015
		Oil	2.3105						
		Saline	3.3062						
		Immersion	2.3314						
6	0.0197	Control	1.1997	0.7967	0.0744	0.1616	0.1252	0.0986	0.0019
		Oil	1.3183						
		Saline	2.0297						
		Immersion	0.5511						
8	0.2	Control	2.319						
		Oil	1.8635						
		Saline	1.9025						
		Immersion	1.6468						
10	<. 0001	Control	3.2836	0.0174	0.0485	0.0242	0.6714	<. 0001	<. 0001
		Oil	3.8683						
		Saline	3.7659						
		Immersion	2.7306						
12	0.0794	Control	0						
		Oil	0.5842						
		Saline	0.7938						
		Immersion	0.5184						

Table 2.1. - continued.

Day	F test (Model)	Method	logEV (avg)	t test (comparisons) C vs. O	C vs. S	C vs. I	O vs. S	O vs. I	S vs. I
14	0.2839	Control	0.3075						
		Oil	0.3112						
		Saline	0.466						
		Immersion	0						
21	0.0042	Control	2.6111	0.8843	0.0127	0.0098	0.0086	0.0065	0.9216
		Oil	2.6751						
		Saline	1.4913						
		Immersion	1.448						
28	<. 0001	Control	2.5236	0.0008	0.7093	0.001	0.0024	<. 0001	0.0003
		Oil	3.6212						
		Saline	2.6406						
		Immersion	1.4503						
35	<. 0001	Control	0.2335	0.0001	0.8463	0.4899	0.0003	<. 0001	0.3772
		Oil	1.4102						
		Saline	0.2906						
		Immersion	0.03						
42	<. 0001	Control	0.3178	<. 0001	0.5045	0.5962	<. 0001	<. 0001	0.2328
		Oil	2.6399						
		Saline	0.5562						
		Immersion	0.1287						
101	0.0151	Control	0.4307	0.0385	0.2408	0.8577	0.0015	0.0578	0.1772
		Oil	1.03						
		Saline	0.0943						
		Immersion	0.4819						
181	0.0014	Control	0.4246	0.0244	0.1155	0.3715	0.0002	0.002	0.4905
		Oil	1.0373						
		Saline	0						
		Immersion	0.1848						
286	<. 0001	Control	2.9962	0.2768	0.0003	<. 0001	0.0081	0.0031	0.7414
		Oil	2.5212						
		Saline	1.3416						
		Immersion	1.1979						
381	<. 0001	Control	0.1039	0.011	<. 0001	<. 0001	<. 0001	0.0032	0.2657
		Oil	1.0438						
		Saline	2.5435						
		Immersion	2.1394						

Figure 2.2. – Early daily variation in kinetics of the serum antibody response of rainbow trout following vaccination. Daily values represent average antibody titers determined by ELISA of twenty fish per vaccination method.



Post-challenge results showed that the overall model, tank, and treatment were significant for all days (Table 2.2). No statistically significant differences in position were observed post-challenge for any days except day 381. Post-challenge comparisons in mean logEV among treatment groups showed the oil-adjuvant injection vaccinated group had a significantly higher mean logEV than the other vaccinated groups on all days except on day 181 (Table 2.3, Figure 2.3). The non-vaccinated groups mean logEV was not significantly different from the saline-based injection vaccinated group or the immersion vaccinated group at days 42 and 101. However, at days 181, 286, and 381 significance was found. The saline-based injection vaccinated groups mean logEV was not significantly different from the immersion vaccinated groups mean logEV for any days.

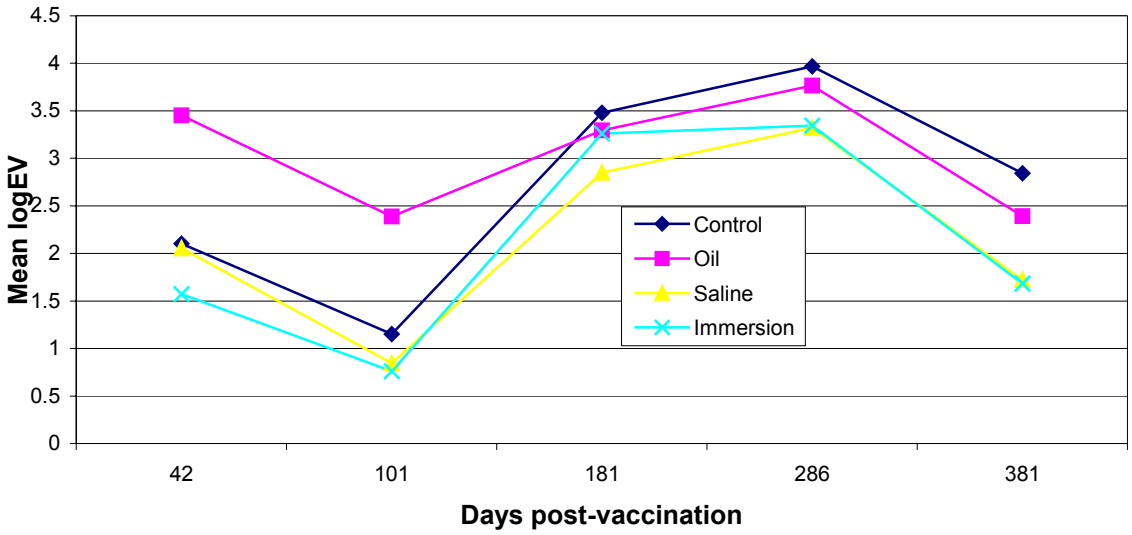
Table 2.2. – Post-challenge ANOVA results of model, tank, position, and treatment effects at each sampling date. Significance is expressed at $\alpha \leq 0.05$.

Day	Pr > F (Model)	Pr > F (Tank)	Pr > F (Position)	Pr > F (Treatment)
42	< .0001	< .0001	0.574	< .0001
101	< .0001	< .0001	0.8687	< .0001
181	< .0001	< .0001	0.1906	0.0021
286	< .0001	< .0001	0.4819	< .0001
381	< .0001	< .0001	0.0004	< .0001

Table 2.3. – ANOVA statistics for post-challenge mean logEV comparisons among treatment groups. Significance is expressed at $\alpha \leq 0.05$. Control (C), Oil (O) - oil adjuvant injected group, Saline (S) – saline-based injected group, Immersion (I) – standard immersion vaccinated group.

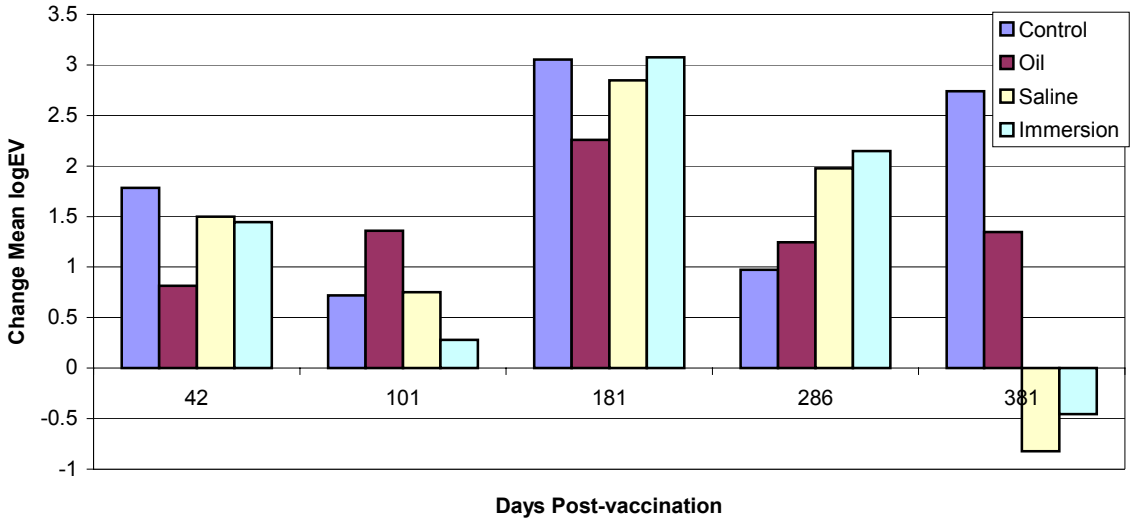
Day	Method	Log EV (mean)	t test (comparisons)					
			C vs. O	C vs. S	C vs. I	O vs. S	O vs. I	S vs. I
42	Control	2.102	< .0001	0.8636	0.0623	< .0001	< .0001	0.0865
	Oil	3.453						
	Saline	2.055						
	Immersion	1.573						
101	Control	1.150	< .0001	0.1915	0.0995	< .0001	< .0001	0.7088
	Oil	2.389						
	Saline	0.846						
	Immersion	0.760						
181	Control	3.479	0.5050	0.0315	0.4283	0.1160	0.8979	0.1455
	Oil	3.295						
	Saline	2.849						
	Immersion	3.260						
286	Control	3.969	0.2107	0.0006	0.0009	0.0107	0.0155	0.8663
	Oil	3.766						
	Saline	3.320						
	Immersion	3.346						
381	Control	2.843	0.1033	0.0005	0.0003	0.0087	0.0062	0.8756
	Oil	2.391						
	Saline	1.721						
	Immersion	1.685						

Figure 2.3. - Post-challenge kinetics of the serum antibody response of rainbow trout following vaccination.



A comparison was also made on the change in mean logEV from pre-challenge to post-challenge (Figure 2.4). Changes varied between challenge days and no overall trends could be determined.

Figure 2.4. - Change in serum antibody titer of rainbow trout from pre-challenge to post-challenge. Fish used for pre-challenge serum samples were not used for *Y. ruckeri* exposure tests.



2.4 Discussion

Antibody titer in rainbow trout after vaccination showed no correlation with vaccination method. The oil-based adjuvant injected group did show increased levels of circulating antibody when compared to the other vaccinated groups however, the validity is not truly known due to the response seen in the non-vaccinated group (Figure 2.3). Statistical significance was observed in position at day 381 only. This may be attributed to simple random processes since day 381 was the only time significance was determined (Table 2.2).

The large discrepancies in response seen in the first days after vaccination could possibly be attributed to individuals responding to the vaccine at different rates (Figure 2.2). Stress from the vaccination process may have also contributed to high antibody

levels during this time. The high titers in the control groups for kinetics (Table 2.1) and post-challenge (Table 2.2) may be associated with cross-reacting antibody. Exposure to other gram-negative organisms may have also induced antibody production (Neumann and Tripp 1986).

Plasma was collected from the surviving individuals in post-challenge comparisons. This could explain why the non-vaccinated group showed unexpectedly high ELISA values when compared to the vaccinated groups. Fish that succumbed to the challenge apparently did not elicit an effective immune response.

The protective antigen, in *Y. ruckeri*, is presumed to be the LPS component of the bacteria. Stevenson (1997) stated that the immune response to O:1 strains of *Y. ruckeri* was negligible or weak compared to that of O:2 strains when tested in Western blots or ELISA's against purified antigens. Olessen (1991) postulated that the antibodies may only interact with antigens in vivo and not in the ELISA trays. If the immune response is to some other antigenic component, or if the antibodies react in vivo only, then the ELISA used in this study would not appropriately measure the response.

Within the parameters of this study, no significant differences in the kinetics of the antibody response to *Y. ruckeri*, regardless of vaccination method, could be detected. Furthermore, no significant differences in elevated circulating antibody levels could be detected in vaccinated fish after bath challenge.

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3. Duration of Protective Immunity and Comparison to ELISA Antibody Titer After Bath Challenge

3.1 Introduction

The potential virulence of *Y. ruckeri* has been extensively studied, largely to determine the efficacy of vaccine preparations (Anderson and Nelson 1974; Busch and Lingg 1975; Bullock et al. 1976; Hunter et al. 1980; Johnson et al. 1982a, Amend et al. 1983, Bosse and Post 1983; Bruno and Munro 1989). These studies, and many more, have resulted in an increased knowledge of the characteristics and mechanisms of the bacteria and improved ways to control disease from *Y. ruckeri*. As an epizootic in hatcheries, ERM constitutes a major economic problem however, ways to minimize outbreaks are available. Cossarini-Dunier (1986) observed only 5.5% mortality after challenge in rainbow trout injected with a *Y. ruckeri* vaccine mixed with saline or oil adjuvant compared to 88.5% mortality in control fish. Experiments using serotype I and II isolates showed brook trout produced specific antibody against the vaccine serotype but were protected against both serotypes when challenged by bath exposure (Cipriano and Ruppenthal 1987).

The purpose of this study was to compare the efficacy of immersion and intraperitoneal injection (saline-based injection and oil-based adjuvant injection) vaccination to a North Carolina isolate of *Y. ruckeri*. Susceptibility to challenge was also compared between vaccinated and non-vaccinated groups. Antibody response and protection were evaluated to determine the preferred method of vaccination.

3.2 Materials and Methods

Challenges

Materials and methods are those as described in 2.2. During challenges, any dead or moribund fish were removed daily. Fish length and weight were recorded and samples from posterior kidney were streaked on TSA plates for isolation of the bacteria. Plates were incubated at 22°C for 48 hours. Confirmation of *Y. ruckeri* colonies was established by macroscopic slide agglutination, oxidase, and catalase tests (Appendix 3.1). Death was assumed to be due to infection by *Y. ruckeri* if positive confirmation of the bacteria was present in the kidney streaks. If *Y. ruckeri* was not isolated, the assumption of death by other causes was made and the mortality was not included in the final count.

Statistical Analysis

Mortality data collected at post-challenge days 42, 101, 181, 286, and 381 was used for analyzing survival of the vaccinated fish. An analysis was performed for each day and overall. The analysis was performed in two stages. The first stage used an ANOVA model with SAS General Linear Models Procedure. A three-factor ANOVA model with percent mortality as the dependent variable and treatment, tank, and position as independent variables was used to determine if significant tank and position effects existed. Statistical significance was determined at an α level of ≤ 0.05.

Since the assumption of normally distributed data was not true and the independent variables of tank and position were insignificant, the second stage of the analysis used Fisher's exact test to test for a significant difference in the number of

deaths for each treatment group. SAS Proc Freq was implemented for Fisher's exact test. The null hypothesis was that vaccination method would not have an effect on mortality versus the alternative hypothesis that vaccination method would have an effect on mortality. More specifically, injection vaccination groups would confer better protection than the immersion vaccination group. Also, the oil-based adjuvant injection group would show better protection than the saline-based injected group.

3.3 Results

Relative percent survival of vaccinated groups ranged from 75% to 100% during all challenges (Table 3.1). An increase in the number of mortalities was observed with increasing temperature with the exception of challenge day 286 (Figure 3.1).

Table 3.1. - Summary of mortality and temperature data for all challenges. Relative percent survival is calculated as: RPS = 1- (% Vaccinated Mortality/% Non-vaccinated Mortality) X 100.

Day 42 Challenge Method	Mortality	Total # fish	% Mortality	RPS	Avg. Temp. (Degrees C) 10.5
Control	4	200	2		
Oil-Injection	1	200	0.5	75	
Saline-Injection	0	200	0	100	
Immersion	0	200	0	100	
Total mortality	5				

Table 3.1. - continued

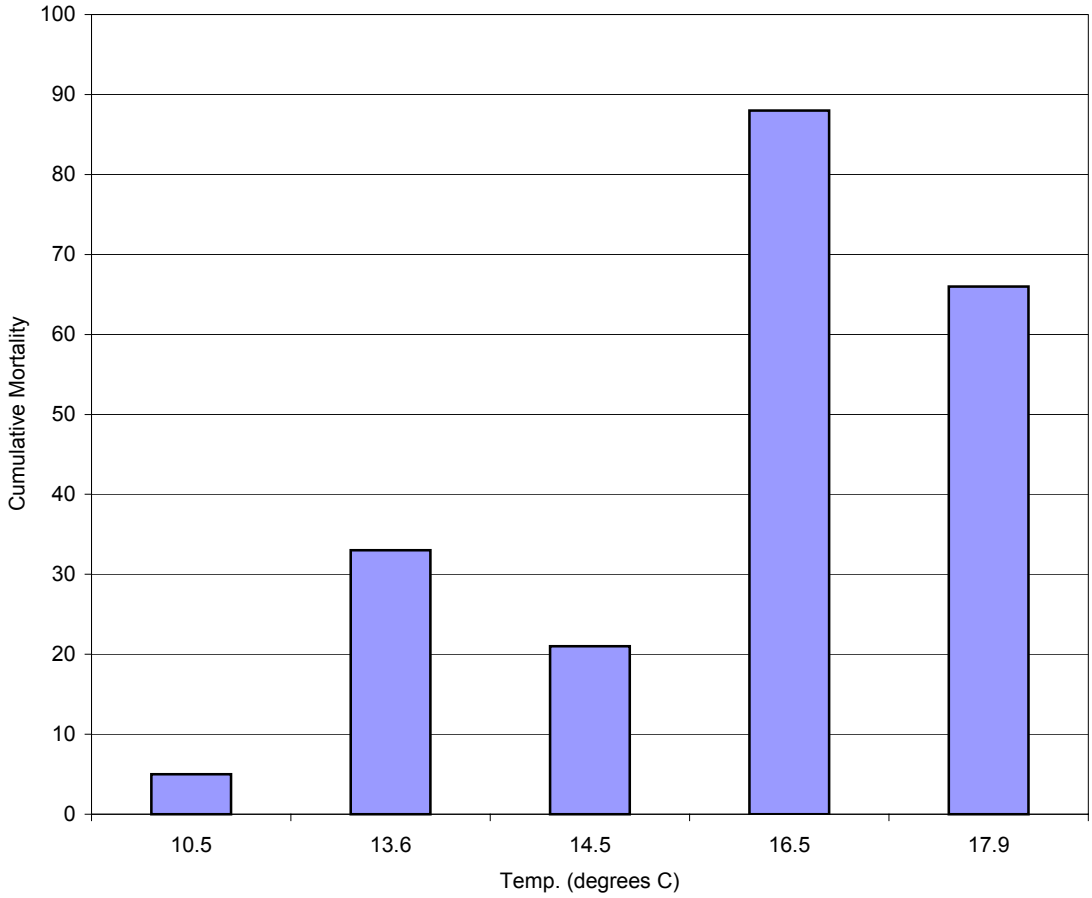
Day 101Challenge Method	Mortality	Total # fish	% Mortality	RPS	Avg. Temp. (Degrees C) 14.5
Control	18	200	9		
Oil-Injection	0	200	0	100	
Saline-Injection	1	200	0.5	94.44	
Immersion	2	200	1	88.89	
Total mortality	21				

Day 181 Challenge Method	Mortality	Total # fish	% Mortality	RPS	Avg. Temp. (Degrees C) 16.5
Control	84	200	42		
Oil-Injection	1	200	0.5	98.81	
Saline-Injection	1	200	0.5	98.81	
Immersion	2	200	1	97.62	
Total mortality	88				

Day 286 Challenge Method	Mortality	Total # fish	% Mortality	RPS	Avg. Temp. (Degrees C) 17.9
Control	63	200	31.5		
Oil-Injection	1	200	0.5	98.41	
Saline-Injection	0	200	0	100	
Immersion	2	200	1	96.83	
Total mortality	66				

Day 381Challenge Method	Mortality	Total # fish	% Mortality	RPS	Avg. Temp. (Degrees C) 13.6
Control	29	80	36.25		
Oil-Injection	0	144	0	100	
Saline-Injection	1	200	0.5	98.62	
Immersion	3	200	1.5	95.86	
Total mortality	33				

Figure 3.1. - Response of mortality to increasing temperature. Day 42 = 10.5°C, Day 101 = 14.5°C, Day 181 = 16.5°C, Day 286 = 17.9°C, Day 381 = 13.6 °C.



ANOVA results on percent mortality showed no tank or position effects in the study however, significance did occur among treatments at each challenge. Comparisons among treatment groups resulted in significant differences in mortality between vaccinated and non-vaccinated groups only. No significant differences were found in comparisons among the vaccinated groups (Table 3.2).

Table 3.2. - Summary of SAS GLM % Mortality Analysis. Means with the same letter are not significantly different. Significance is expressed at $\alpha \leq 0.05$. Treatments = 1- Control Group, 2- Oil-based Adjuvant Injection Group, 3- Saline-based Injection Group, 4- Immersion Group.

Day	F test (tank)	F test (position)	F test (trt)	t Grouping	Mean	Treatment
42	0.6986	0.1553	0.0166	A	2	1
				B	0.5	2
				B	0	3
				B	0	4
101	0.4065	0.0600	<. 0001	A	9	1
				B	0	2
				B	0.5	3
				B	1	4
181	0.5468	0.1189	<. 0001	A	42	1
				B	0.5	2
				B	0.5	3
				B	1	4
286	0.3570	0.1150	<. 0001	A	31.5	1
				B	0.5	2
				B	0	3
				B	1	4
381	0.5058	0.2829	<. 0001	A	35	1
				B	0	2
				B	0.5	3
				B	1.5	4

Analysis of the individual mortality counts was done using Fisher's Exact Test. This test allows for analyses to be conducted when actual cell counts are low, as is the case with the low numbers of mortalities in the vaccinated groups. These results further support the analysis using the GLM procedure (Table 3.3).

Table 3.3. - Fishers Exact test for mortality. Significance is expressed at $\alpha \leq 0.05$.

Comparisons	P value
Treatments	<. 0001
Vaccinated Groups	0.1249
Control vs. Vaccinated	<. 0001
Control Vs. Oil	<. 0001
Control vs. Saline	<. 0001
Control vs. Immersion	<. 0001
Oil vs. Saline	1
Oil vs. Immersion	0.1468
Saline vs. Immersion	0.1448

The saline-based injection group showed the greatest degree of protection (98.96% survival) followed by the oil-adjuvant injection group (98.89% survival) and lastly, the immersion-vaccinated group (96.85% survival) (Table 3.4). This suggests that vaccination is key to fish survival when exposure to *Y. ruckeri* is likely to occur.

Table 3.4. - Frequency Table of Treatment by Alive.

Treatment	Alive	Dead	% Mortality	RPS
Control	683	197	28.84	
Oil	941	3	0.32	98.89
Saline	997	3	0.30	98.96
Immersion	991	9	0.91	96.85

3.4 Discussion

The null hypothesis that vaccination method would not have an effect on mortality was accepted. The alternative hypothesis that injection vaccination confers better protection than immersion vaccination and that an oil-based adjuvant would protect

fish better than a saline-based vaccine was not proven. Protection was conferred in all vaccinated groups with no significant differences in survivability. This demonstrates all fish were equally protected regardless of vaccination method (Tables 3.2, 3.3, 3.4). An increase in mortality was observed with an increase in temperature with the exception of challenge day 286. Similar studies have shown the optimal temperature for *Y. ruckeri* growth is 15-18°C (Ellis 1988).

The findings of this study are in agreement with several other studies that have suggested that relatively high levels of antibodies to *Y. ruckeri* are not required for protection (Michel and Faivre 1982; McCarthy et al. 1983; Olivier et al. 1985; Cossarini-Dunier 1986; Neumann and Tripp 1986; Cipriano 1987; Olesen 1991; Toranzo et al. 1995). Croy and Amend (1977) demonstrated protection in coho salmon against *Vibrio anguillarum* by immersion with no significant differences in antibody titers among test and control fish. In order for any vaccine to elicit a protective immune response, an appropriate immune response must be induced (Thune et al. 1997). Non-specific humoral or cellular factors such as complement, lectins, macrophages, granulocytes, etc. are important secondary lines of defense. Of these, complement seems to be the most important. Complement has the ability to kill pathogens as well as increase phagocytosis by macrophages, migration of leukocytes, and production of antibodies (Nakanishi and Ototake 1997). The present study shows that the immune response elicited after bath challenge may be enough to sufficiently protect the fish even though the difference in antibody response of surviving vaccinated and non-vaccinated fish was not significantly different.

The mucus and epidermis are also important barriers in the defense mechanisms of fish to pathogens. Sakai et al. (1989) showed an increase in the phagocytic activity of kidney leukocytes after direct immersion in a streptococcal bacterin. The presence of a mucosal immune system has been demonstrated in many fish species (Kawai et al. 1981; Lobb and Clem 1981; Rombout et al. 1986; Davidson et al. 1993). A defense system in the skin rather than humoral or mucosal has also been suggested for eels (Mano et al., 1996). Enhanced skin mucus titers, rather than serum titers, may explain why differences in antibody levels and RPS, among vaccinates, was not significant in the present study. If the response to the bacteria was generated at the skin surface, immersion vaccination may be the most appropriate vaccination method since vaccine exposure will most likely occur via the same route as natural bacterial exposures.

Studies have shown immunity in salmonids increased with size (range 1-4g) suggesting that even though immunity is detected in smaller (younger) fish, immune memory may take longer to develop. Also, 6.3g fish had lower mortalities than 4.1g fish that had been exposed to *Vibrio anguillarum* after vaccination further supporting maturation of immune protection with age and/or size (Johnson et al. 1982 a and b; Thorburn and Jansson 1988). Immersion vaccines are typically administered at 3.5-5.0 g to ensure protection (Horne 1997). Vaccinating large numbers of fish by injection is difficult until they grow to approximately 15-20 g. This study indicates fish at approximately 25 g are equally protected regardless of the vaccination method used.

The importance of humoral factors in immune processes is still not completely understood. Inconsistencies in the literature on the correlation of protection to circulating antibody levels further add to this problem. Though no significant antibody responses

were detected in the present study, all vaccinated groups were protected. Humoral immunity may still be necessary for protection. Future studies will need to focus on the specific defense mechanisms involved in humoral and cell-mediated immunity. Increased knowledge of the fish immune response will increase development of more effective vaccines. A more complete understanding of the size at which immersion vaccination is most effective also needs to be addressed.

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Appendix 2.1. – Preparation of LPS for use in ELISA.

Propagation of the bacterial culture was done by removing a capillary tube containing *Y. ruckeri* from ultra-cold storage and transferring the bacteria to a tube containing 7 ml sterile TSB. The tube was incubated overnight at 26°C. Two ml of the 24-hour bacterial suspension was added to each of 2 tubes containing 15 ml sterile TSB and incubated overnight at 22°C. Finally, 7.5 ml of this bacterial suspension was added to 4-500 ml sterile Erlenmeyer Flasks containing 250 ml sterile TSB and incubated, with shaking, overnight at 22°C. The bacterial suspensions in the flasks were transferred to 50 ml centrifuge tubes. The tubes were centrifuged for 10 minutes at 500 x G at 25°C. The supernatant was pipetted off and the bacterial cell packs were rinsed with sterile saline and centrifuged for 10 minutes at 500 x G at 25°C. The saline supernatant was pipetted off and the bacterial cell packs were resuspended in sterile saline and combined. The suspension was adjusted to approximately 10^9 cells/ml using McFarland Standards #3 (9 x 10^8 cells/ml) and #4 (1.2 x 10^9 cells/ml). The suspension was autoclaved at 120°C for 30 minutes then transferred to centrifuge tubes and centrifuged at 500 x G for 15 minutes. The supernatant (LPS) was pipetted off and stored in aliquots of 0.5-1.0 ml at -80°C.

Appendix 2.2. - Affinity purification of rainbow trout Ig.

Rainbow trout Ig was isolated using a mannon-binding protein (MBP) affinity column. The column was washed with 5 ml ImmunoPure MBP column preparation buffer and equilibrated with 20 ml of ImmunoPure IgM binding buffer at 4°C. Fish serum was diluted 1:2 with ImmunoPure IgM binding buffer and dialyzed overnight at 4°C in a tris-NaCl buffer. One ml of dialyzed serum was applied to the column. After 30 minutes, the column was washed with binding buffer to remove unbound protein. The column was then placed at room temperature and 3 ml of ImmunoPure IgM elution buffer was added and allowed to incubate for 1 hour. Three ml fractions were collected and the protein content was measured. Eluates with significant amounts of protein (>10μg/ml) were pooled , concentrated, and desalted by centrifugation. Purified Ig was stored at -70°C in PBS.

Appendix 2.3. - Production of rabbit anti-trout Ig antibodies.

Polyclonal antibodies were produced in rabbits by the RIBI Adjuvant System (RAS). The RAS formulation was reconstituted in 2 ml of 0.15 M PBS containing 200 μg of affinity-purified Ig and warmed to 37°C prior to use. Each rabbit was anesthetized (Day 0) with acepromazine and butorphanol and 4 ml of whole blood was collected. One ml RAS was then injected as follows: 6 intradermal sites in the neck (0.05 ml); 1 intramuscular site in each hind leg ((0.3 ml); 1 subcutaneous site in the neck (0.1 ml). Rabbits were boosted on Day 21 and test bled on Day 28. Exsanguination was performed under anesthesia on Day 31 using ketamine hydrochloride. All sera were stored at -70°C.

Appendix 2.4. - Procedure for conduction of ELISA.

LPS derived from the original *Y. ruckeri* isolate was diluted 1:500 in coating buffer* (0.4g Na_2CO_3, 0.73g $NaHCO_3$, 250 ml dH_2O, pH 9.6) and applied to 96 well ELISA plates (Corning). After incubation at 37°C for one hour, plates were washed (4X) in a saline solution (8.5g NaCl, 0.5 ml Tween 20, 1 L dH_2O). Blocking buffer (4.3g NaCl, 0.18g EDTA, 3.0g Tris, 500 ml dH_2O, pH 7.4 then 1.5g Bovine Serum Albumin (BSA)) was then added (200µL/well) and incubated at 25°C for 45 minutes. After washing, as above, test and control negative and control positive fish plasma diluted 1:200* in Tris buffer (as per blocking buffer except 0.5g BSA and 0.25 ml Tween 20) was dispensed (100µL/well) in triplicate on each plate. A blank sample containing Tris buffer alone was also included on each plate. Plates were incubated at 25°C for 45 minutes then washed (4X). Next, rabbit anti-trout antiserum (12µl rabbit anti-trout, 12 ml Tris buffer, 100 µL thawed *Y. ruckeri* LPS, incubated at 37°C for one hour with mixing), prepared as previously described by Cobb et al. (1998), was added (100µL/well) to each plate and incubated at 25°C for 45 minutes then washed (4X). Conjugate (Peroxidase-labelled goat anti-rabbit IgG (H+L), Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added (100µL/well) and the plates were incubated at 25°C for 45 minutes. After washing (4X), the substrate (TMB Microwell Peroxidase Substrate System) was added (100µL/well) for 3 minutes. A stop solution of 10% phosphoric acid (100µL/well) was added to complete the ELISA procedure. The plates were read using a Fisher Biotech BT2000 MicroKinetics microplate reader at 450nm.

Appendix 2.4. - Continued.

After zeroing with the blank sample, average optical densities (OD) of the triplicate samples were converted to ELISA (EV) values by:

$$\frac{\text{Test fish plasma OD} - \text{Negative control plasma OD}}{\text{Positive control plasma OD} - \text{Negative control plasma OD}} \times 100 = \text{EV}$$

* LPS was reoptimized to a dilution of 1:1000 and the fish plasma was reoptimized to a dilution of 1:500 upon the acquisition of a new lot of peroxidase goat anti-rabbit conjugate.

Appendix 3.1. – Procedure for macroscopic slide agglutination test.

1. On a clean ring slide:
    a. Place 1-2 drops of sterile saline in the first well. This will be the control.
    b. Place 1-2 drops of the bacteria specific antiserum in all other wells being used. These will be the test wells.
2. Using a sterile inoculating loop, remove a small amount of the bacterial sample to be tested and place in each well.
3. Gently swirl to mix.
4. Observe reaction over a 5-10 minute period.
5. Positive detection is confirmed by agglutination in the test wells (b.) and no agglutination in the control well (a.).