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Immunization of Rainbow Trout (*Oncorhynchus mykiss*) against *Lactococcus garvieae* Using Vaccine Mixtures

A. Kubilay[1]*, S. Altun[1], G. Ulukoy[2], S. Ekici[1] and O. Diler[1]

[1] *Department of Aquaculture, Faculty of Fisheries, Suleyman Demirel University, 32500 Egirdir, Isparta, Turkey*

[2] *Department of Aquaculture, Faculty of Fisheries, Mugla University, 48000 Kotekli, Mugla, Turkey*

Abstract

The effectiveness of vaccine mixtures against lactococcosis was tested in rainbow trout (*Oncorhynchus mykiss*). The M_1 strain of *Lactococcus garvieae*, isolated from a recent outbreak of lactococcosis at a rainbow trout farm in Turkey, was used in a trial comparing five immunization treatments: (a) formalin inactivated bacterin (vaccine), (b) the above bacterin together with Freund's Incomplete Adjuvant (FIA), (c) the bacterin combined with β-glucan, (d) β-glucan only, and (e) phosphate buffered saline-PBS (control). Fish were given intrapritoneal injections and challenged by exposure to the bacteria 30, 75, or 125 days after vaccination. In fish exposed to the bacteria 30 days after injection, the relative percent survival (RPS) was 88.89% in the group that received only bacterin and 100% in the group that received the bacterin combined with FIA. Immunity remained high in the bacterin+FIA group, as the RPS in this group remained 100% in fish challenged at 75 days, significantly higher than in all other groups. In fish exposed to the bacteria 125 days after vaccination, the RPS was 54.55% in fish vaccinated with the bacterin only and 84.84% in fish vaccinated with bacterin+FIA. In the group that received only β-glucan, immunity did not improve after vaccination. Micro-agglutination tests of serums showed that immunized fish produced antibodies at high titers within 30 days. In short, the formalin-inactivated M_1 strain provided longer lasting protection against *Lactococcus garvieae* in rainbow trout when combined with FIA than when administered alone or with β-glucan.

Introduction

Lactococcosis is a worldwide septicemic fish disease characterized by bilateral exophthalmia and caused by the bacteria *Lactococcus garvieae* (Austin and Austin 1999; Kusuda and Salati, 1999). A variety of fish species can be affected by this disease (Eldar and Ghittino, 1999; Diler et al., 2002; Salati et al., 2005). Lactococcosis infection is

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* Corresponding author. Tel.: +90-246-3133447, fax: +90-246-3133452, e-mail: aykub@ yahoo.com

the single most important risk factor in the European trout industry, with losses approximating 50% of the total annual production (Eyngor et al., 2004). The impact of lactococcosis is particularly significant as losses often occur when fish reach market size (Ceschia et al., 1998).

In Turkey, *L. garvieae* was first isolated in 2001 after an outbreak on rainbow trout farms (Diler et al., 2002; Altun et al., 2004). Since then, infections have been repeatedly occurring, especially during the warm summer months. *Lactococcus garvieae* is now considered one of the most important pathogens in Turkey's rainbow trout industry.

Lactococcus garvieae is a gram-positive coccus. It is oxydase and catalase negative, non-motile, and a facultatively anaerobic bacteria. Antimicrobials have not been successful against *L. garvieae* as resistance is rapidly developed. Thus, the only possible means of control is vaccination (Romalde et al., 2004; Salati et al., 2005).

Among the commercially available bacterins against bacterial fish pathogens, none exist for *L. garvieae* (Romalde et al., 2004). Production of lactococcosis vaccines have been attempted (Bercovier et al., 1997), also with the use of other gram-positive pathogens (Eldar et al., 1997; Romalde et al., 1999). However, the short duration of immunity (two or three months) limits the success of these vaccines since the immunity does not persist throughout the entire warm season when the majority of lactococcosis outbreaks occur (Bercovier et al., 1997; Romalde et al., 2004; Ravelo et al., 2006).

The aim of this study was to test the efficacy and lasting level of a formalin-killed vaccine alone and together with immunostimulants against lactococcosis in rainbow trout.

Materials and Methods

Fish. Juvenile rainbow trout were obtained from a farm with no history of lactococcosis and acclimated for six weeks. The fish were fed commercial pellets at 2% body weight per day.

Selection of the bacterial strain. The vaccine was prepared from the M_1 strain of *L. garvieae* isolated during a natural outbreak that occurred in a rainbow trout farm in Fethiye-Mugla, Turkey (Diler et al., 2002). This strain was selected among several strains on the basis of antigenic characteristics determined in earlier studies (Altun et al., 2004, 2007). The reference strain NCDO 2155 (ATCC-43921) was compared with the isolated strain after inoculation on trypticase soy agar (TSA, Difco Laboratories, Detroit, MI) and incubation at 25°C for 24-48 h. Pure bacteria were transferred to tryptic soy broth (Difco Laboratories, Detroit, MI) and pure culture stocks were stored at -80°C in tryptic soy broth (TSB, Difco) containing 15% glycerol.

Immunostimulant and adjuvant. Freund's Incomplete Adjuvant (FIA; Sigma Chemical Co., St Louis, MO, cat. no. F-5506) and β-glucan (Sigma, cat. no. G-6513) were used as immunostimulants.

Enhancing the virulency of the M_1 strain. *In vivo* passage was used to increase the virulency of the *L. garvieae* M_1 strain (Eldar et al., 1995). The LD_{50} of the M_1 strain was calculated by determining the cumulative mortality rates one month after injection of M_1 strain at two doses.

Preparation of the bacterin. A formalin-killed bacterin was prepared from the selected strain according to Eldar et al. (1997). The strain was grown in TSB for 24 h and the bacterial cells were inactivated by adding formalin until a final concentration of 0.7% was obtained. The solution was incubated at 25°C for 3 h and then overnight at 4°C. Thereafter, the inactivated bacterial cells were washed three times with phosphate buffered saline (PBS) by centrifugation at 6000 x *g* for 30 min at 4°C. The formalin-killed vaccine was resuspended in PBS and adjusted by optical density to a final concentration of about 1×10^{11} CFU/ml (OD_{600} of 1.2)

Immunization. Six hundred fish (avg wt 20 g) were divided into ten groups and two groups were injected intraperitonally (i.p.) with each of the following five treatments: (a) 0.1 ml bacterin at a concentration of 1×10^{10} CFU/ ml, (b) the same amount of bacterin plus Freund's Incomplete Adjuvant (FIA) at a ratio of 1:1, (c) the same amount of bacterin plus β-

glucan, (d) β-glucan alone at 2 mg/fish, or (e) phosphate buffered saline (control). Fish were anesthetized using quinaldine (1/20,000) prior to injection. Fifty fish in each group were challenged as specified below to determine relative percent survival and 10 were examined in a micro-agglutination assay to determine antibody titers.

Fish were kept in 600-l tanks with an open flow of fresh water at 1-1.5 l/min and constant aeration. Water temperature was kept at 12°C. After vaccination fish were fed commercial pellets (extruded feed no. 3, 45% protein, 20% lipids, Kilic Feed, Mugla, Turkey) at 2% body weight per day.

Challenge tests and determination of vaccine potency. Fish were challenged 30, 75, and 125 days after immunization with a homologous strain of *L. garvieae* prepared as above and individually injected at 0.1 ml/fish. Mortality was recorded daily for three weeks after challenge. Internal organs of all dead fish were examined to confirm infection by re-isolating the inoculated strain. The efficacy of the vaccine in each trial was assessed by calculating the relative percent survival (RPS; Amend, 1981) using the formula: RPS = 1 - (% mortality in vaccinated fish/% mortality in control) x 100.

Serum samples. At 30, 75, and 125 days post-vaccination, ten fish from each group (twenty from each treatment) were briefly anesthetized and bled by dorsal aorta puncture to collect serum for determination of antibody titer. Blood samples were centrifuged at 3000 rpm for 10 min. The serum was collected and aliquots of the serum were kept at -20°C until required for assay.

Micro-agglutination tests. Serial two-fold dilutions of the serum samples in PBS were added to round-bottom polystrene, 96-well, microplates in triplicate for the microagglutination tests. Each reaction contained 100 μl of the M_1 strain of *L. garvieae* at a concentration of 1.2 x 10^9 CFU/ml. The bacterium was inactivated with formalin. The microplates were shaken and incubated at 37°C for 2 h and left overnight at 4°C. The appearance of a button with fuzzy edges at the bottom of the well was considered a positive reaction, the formation of a round precipitate with sharp contours was considered a negative reaction (Eldar et al., 1997; Barnes et al., 2002).

Statistical analysis. Results were analyzed by ANOVA and significances were detected by Duncan test (SPSS 9.0 package for Windows).

Results

The bacteria produced 50% cumulative mortality at a concentration of 4.69 x 10^7 CFU/ml.

Protection levels (resistance or strength of the immunological response) after injection are given in Table 1. Vaccines containing bacterin resulted in greater levels of protection than the control, indicated by the lower mortality and higher RPS in fish exposed 30 days after vaccination. Protection remained high over time as significant differences in mortality were observed between the control and the treatment groups even when fish were exposed to the bacteria 75 and 125 days after vaccination. The statistically highest level of protection was achieved in rainbow trout that received the bacterin+FIA vaccine. In addition, no side effects were observed other than internal organ adhesion. Fish inoculated with β-glucan alone did not benefit from improved protective immunity.

Serum agglutinating antibody titers are given in Table 2. Independent of the vaccination regime, the average antibody titers against formalin-killed antigens of the M_1 strain of *L. garvieae* employed in the vaccine formulation were 1/512 within 30 days after immunization. In addition, an increase in circulating specific antibodies was observed in fish vaccinated with bacterin plus FIA or β-glucan. Therefore, the humoral response correlated with the degree of protection conferred by the vaccine.

Discussion

Good protection levels are achieved only when vaccines are intraperitoneally administered (Bercovier et al., 1997). Immersion procedures produce a lesser degree of protection against gram-positive bacterial infections in salmonids (Bercovier et al., 1997; Romalde et al., 2004). In our study, RPS was 88.89%

Table 1. Mortality (%) and relative percent survival (RPS; %) in rainbow trout (*Oncorhynchus mykiss*) 30 days after challenge* with *Lactococcus garvieae* on day 30, 75, or 125 after immunization (n = 100/treatment).

Immunization group	Challenge on day					
	30		75		125	
	Mortality	RPS	Mortality	RPS	Mortality	RPS
Bacterin	6c	88.89	14c	76.67	30bc	54.55
Bacterin + Freund's Incomplete Adjuvant	0d	100	0e	100	10c	84.84
Bacterin + β-glucan	6c	88.89	6d	90	30bc	54.55
β-glucan	44b	18.52	50b	16.66	60b	9.09
Phosphate buffered saline (control)	54a	-	60a	-	66a	-

Different superscripts in a column indicate significant differences (*p*<0.05) between groups.
* challenge dose was LD_{50} = 4.69 x 10^7 CFU/ml

when trout were exposed to *L. garvieae* 30 days after i.p. administration of the prepared bacterin only, similar to other results of i.p. immunization of fish, e.g., 90.0% RPS against *Streptococcus iniae* (Eldar et al., 1997), 83.3% RPS against *L. garvieae* (Romalde et al., 2004)*,* and 82.6% RPS against *L. garvieae* (Ravelo et al., 2006). These results indicate a good initial level of protection but a very short duration of immunity (approximately 3 months; Bercovier et al., 1997). Likewise in our study, the initial level of protection was relatively good (88.89% RPS) but dropped to 54.55% RPS after 125 days (post-vaccination).

Romalde et al. (2004) studied the efficacy of oral immunization using alginate-microparticles for a booster vaccination in rainbow trout to prevent lactococcosis. In fish that received an initial i.p. vaccination with aqueous bacterin and an oral alginate-encapsulated booster vaccine 90 days later, protection reached 87% RPS 30 days after revaccination.

Another approach to increasing vaccine potency and/or duration of protection, without revaccination, is to use adjuvants in the vaccine formulation (Anderson, 1997). Ravelo et al. (2006) investigated the use of adjuvanted vaccines to lengthen protection against lactococcosis in rainbow trout. Oil preparations have been used with a high degree of success to prevent a number of bacterial fish diseases (Anderson, 1992; Newman, 1993; Rahman et al., 2000). The use of oil-based adjuvants has now been successfully combined with injectable bacterins in fish. The oil-adjuvants generate a 'depot effect', causing the antigen to be slowly released into the tissue or blood, enhancing and prolonging the humoral response (Anderson, 1997). In the present study, we evaluated the addition of an adjuvant to the vaccine formulation. A significantly higher level of protection (100% RPS) was initially obtained (on days 30 and 75) in fish immunized with bacterin+FIA and the duration of the immulogical protection was extended, reaching an RPS of 84.84% 125 days after immunization.

A number of studies examined the role of

Table 2. Agglutinating antibody titer in immunization groups and control (1/x) (n = 20/treatment).

Immunization group	Day 30	Day 75	Day 125
Bacterin	512	256	32-64
Bacterin + Freund's Incomplete Adjuvant	512-1024	512	256
Bacterin + β-glucan	512-1024	256	16-64
Phosphate buffered saline (control)	2	2	2

glucans as immunostimulants during immune system challenge, in particular, their role in non-specific defense against disease. In our study, we examined the effect of β-glucan on the activation of the specific and non-specific immune system. Although β-glucan has been shown to enhance non-specific defense mechanisms, this stimulation did not correlate with improved protection in rainbow trout infected with *L. garvieae* in our study as the percent mortality in fish vaccinated with β-glucan did not differ than in the unvaccinated control. Toranzo et al. (1995) obtained similar results in turbot infected with *Enterecoccus* sp.

In conclusion, our results suggest that protection against *L. garvieae* in rainbow trout is attained up to 125 days when the bacterin is formulated from inactivated whole cells and combined with FIA, but not β-glucan, and administered intraperitoneally. Our test vaccines were prepared in multiple batches and results were consistent regardless of the batch used to immunize the test fish. These results will help in developing an appropriate immunization program to prevent lactococcosis outbreaks in rainbow trout aquaculture operations. Fish farms with a short production cycle (less than 6 months) could benefit from this adjuvanted vaccine.

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