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Immunogenic and Antigenic Profiles of Nine *Lactococcus garvieae* Strains from Different Rainbow Trout Farms

S. Altun[1], A.K. Adiloglu[2], A. Kubilay[1], O. Diler[1], N. Delibas[3] and R. Sutcu[3]

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

[2] Department of Medical Microbiology, Faculty of Medicine, Suleyman Demirel University, 32260 Isparta, Turkey

[3] Department of Medical Biochemistry, Faculty of Medicine, Suleyman Demirel University, 32260 Isparta, Turkey

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Abstract

The aims of this study were to determine differences with respect to immunogenic potency in the antigenic profiles of nine *Lactococcus garvieae* strains from Turkey, Spain, and England, to develop a bacterin, and to examine the immunological response of rainbow trout to the bacterin. The strains had identical Western blot patterns with 30, 37, 40, 46, 52, and 66 kDA mw protein bands. After culturing the bacteria, proteins were separated by SDS-PAGE and transferred to Immobilon membranes. The membranes were incubated with hyperimmune rabbit sera obtained by immunizing a rabbit against *L. garvieae*. One group of 50 fish was immunized with formalin-killed bacterin prepared from the most immunogenic strain of *L. garvieae*. A second group of 50 fish served as an unimmunized control. Four weeks after vaccination, both groups were challenged intraperitoneally with a homologous strain. The protection rate of the bacterin was judged by the relative percent survival (RPS) of the groups. A significantly high level of protection was achieved in the vaccinated group (88.8% RPS).

Introduction

The gram-positive bacterium *Lactococcus garvieae* is an important pathogen that causes septicemia and meningoencephalitis in freshwater and marine fish (Eldar et al. 1996; Barnes et al., 2002). It has been isolated from cultured species such as yellow tail (*Seriola quinqueradiata*) in Japan and rainbow trout (*Oncorhynchus mykiss*) in Europe and Australia (Kusuda et al 1991; Eldar et al., 1996; Bercovier et al., 1997; Eldar and Ghittino, 1999; Ravelo et al., 2003), as well as from homeothermic and poikilothermic ani-

* Corresponding author. Tel.: +90-246-3133447, fax: +90-246-3133452,
e-mail: soneraltun2003@yahoo.com

mals such as cows, buffalos, the freshwater prawn *Macrobrachium rosenbergii* (Collins et al., 1984; Teixeira et al., 1996; Chen et al. 2001), and humans (Elliott et al., 1991; Fefer et al., 1998). In Turkey, *L. garvieae* was first isolated from rainbow trout farms in 2001 (Diler et al., 2002; Altun et al., 2004). Since then, it has spread rapidly, causing substantial economic losses, and is one of the important pathogens in the trout industry, causing infections especially in summer.

Numerous commercially available vaccines against fish pathogenic bacteria produce good results. However, chemotherapeutic agents such erythromycin, oxytetracycline, and enrofloxacine may be sensitive in *in vitro* susceptibility tests but ineffective *in vivo* (Austin and Van Pouce, 1993; Bercovier et al., 1997), probably due to anorexia of infected farmed fish. Pathogenic properties of antigenic variations of *L. garvieae* isolated from yellow tail in Japan have been described (Alim et al., 1996; Yoshida et al., 1997; Ooyama et al., 2002). In several attempts to develop vaccines against *L. garvieae*, only intraperitoneal administration achieved effective responses (Bercovier et al., 1997). Therefore, it is necessary to test adapted solutions on local strains of the aquatic pathogen *L. garvieae* (Romalde et al., 2004).

The aim of this study was to determine molecular weights of proteins in *L. garvieae* from different farms by analyzing extracted bacterial proteins by blotting with anti-*L. garvieae* antibodies from a rabbit immunized with *L. garvieae* bacterin. One of the advantages of whole-cell protein profile analysis over conventional physiological tests is that once bacteria are isolated and identified to the genus level, proteins can be prepared and SDS-PAGE results can be determined within one day. In contrast, physiologic tests for species identification can require incubation of at least seven days (Elliott et al., 1991). We also aimed to develop a bacterin and examine the immunological response of rainbow trout to the bacterin.

Materials and Methods

Fish. Rainbow trout (initial wt 20 g) were held in 600-l tanks filled with 12°C fresh water at a flow rate of 1-1.5 l/min and continuous aeration. Fish were fed daily at 2% of their body weight and water quality was monitored daily.

Bacterial strains, virulent strains, hyperimmune serum. Nine isolated *L. garvieae* strains from different trout farms were studied (Table 1). The reference strain NCDO-2155 served as a control. Isolated strains were incubated on trypticase soy agar (TSA; Difco Laboratories, Detroit, MI) at 25°C for 24-48 h and pure cultures were stored at -80°C in tryp-

Table 1. Origin of *Lactococcus garvieae* strains.

No.	*Strain*	*Origin*	*Year of isolation*	*Comments*
1	A_3	Antalya, Turkey	2001	
2	B_1	Isparta, Turkey	2003	
3	279-00	Spain	2000	
4	00-21	England	2000	
5	NCDO-2155 (ATCC-43921)	UK	1973	Control
6	K_1	Konya, Turkey	2001	
7	C_1	Mugla, Turkey	2001	
8	M_1	Mugla, Turkey	2001	Virulence experiment
9	D_1	Denizli, Turkey	2001	

ticase soy broth (Difco Laboratories, Detroit, MI) with 15% glycerol. *Lactococcus garvieae* M_1, cultivated from rainbow trout in Turkey, is the most virulent strain (80% loss rates) and was used for the virulence experiment (Diler et al., 2002). LD50 was calculated by determining the cumulative mortality rates one month after injection of M_1 bacteria at two doses. *Lactococcus garvieae* (NCDO-2155) hyperimmune serum was obtained from the Department of Fish Disease in Ege University.

Electrophoresis (SDS-PAGE) and Western blotting. Each strain was sonicated 5 min at maximum amplitude (Bandelin Electronic UW2070, Germany) and centrifuged 30 min at 14,000 x *g* at 4°C. The resulting pellet was stained with Gram stain. Detection of gram-negative cocci indicated that the bacteria underwent complete lysis and that the supernatant consisted predominantly of cell wall components. The supernatant was precipitated by adding five volumes of ice cold acetone. The amount of protein was determined in sonicated bacteria by the technique of Bradford (1976) and treated according to the method of Laemmli (1970).

Equal amounts of protein from each strain (30 µg of protein per lane) were loaded in 10% SDS-PAGE minigels. A constant power of 100 mA was applied to the stocking gel, and a constant voltage of 200 mA was applied to the running jel. Gels were blotted onto Immobilon membrane using a Miniprotean Trans Blotter (EC120 minivertical gel system) as described by Towbin et al. (1979). The blot was incubated in tris-buffered saline with Tween 20 (TBST; 50 mm Tris-HCl [pH 7.5-8.0], 150 mM NaCl, 0.1% Tween 20) containing 3% bovine serum albumin (BSA) for 30 min, then overnight with sera obtained from hyperimmune rabbit (1:100) in a blocking buffer containing 5% skimmed milk. Blots were then subjected to three additional 10-min washings in TBST and incubated with alkaline phosphatase conjugated monoclonal anti-rabbit IgG (1:250) in the same buffer for 1 h at room temperature. The blots were washed three additional times in TBST for 10 min, each. The membrane was incubated in 20 ml of fresh reagent solution (BCIP-NBT) until color developed. Images of immunoblots were analyzed with a computerized image analysis system (Uviphoto MW V.99, Ultra-Violet Products Ltd, Cambridge, UK). Sera from control fish (trout injected intraperitoneally with a saline solution) served for control blots. SDS-PAGE and Western blot analyses were triplicated on all nine strains.

Preparation of bacterin. A formalin-killed bacterin was prepared as described by Eldar et al. (1997). Briefly, after *L. garvieae* strains were incubated in trypticase soy broth for 24 h, the bacteria were killed by adding formalin to a final concentration of 0.7% (v/v). The product was incubated at 25°C for 3 h and at 4°C overnight. The formalin was removed by dialyzing three times with phosphate buffered saline (PBS, pH 7.4) and the pellet was resuspended in PBS at final concentration of 10^{11} cells/ml.

Immunization and challenge of fish. To evaluate the protective effects of the bacterin, two groups of 50 fish (avg wt 20 g) were injected intraperitoneally, one with 0.1 ml of formalin-killed bacterin (10^{10} cells/fish) and one with only PBS. Four weeks after the vaccination, fish from both groups were injected intraperitoneally with homologous strains of *L. garvieae*. Mortality was recorded daily for four weeks and internal organs of all dead fish were examined to confirm infection by reisolating the bacteria. Bacterin protection was evaluated by determining the relative percent survival (RPS; Amend, 1981). The significance of differences in mortality was assessed by chi-square test (GraphPad InStat™). Probabilities lower than 0.01 were considered significant.

Results

The total cell protein profiles of the nine isolated *L. garvieae* strains yielded identical Western-blot patterns with 30, 37, 40, 46, 52, and 66 kDa mw protein bands (Fig. 1). In the virulence test, a dose of 3.76 x 10^{10} cfu/ml bacteria caused 90-94% cumulative mortality in a logarithmic curve analysis while a dose of 5.45 x 10^6 caused only 26-32% cumulative mortality. Statistically significant (p>0.0001) protection was achieved when 6.81 x 10^9 cfu bacterin/ml was administered to immunized

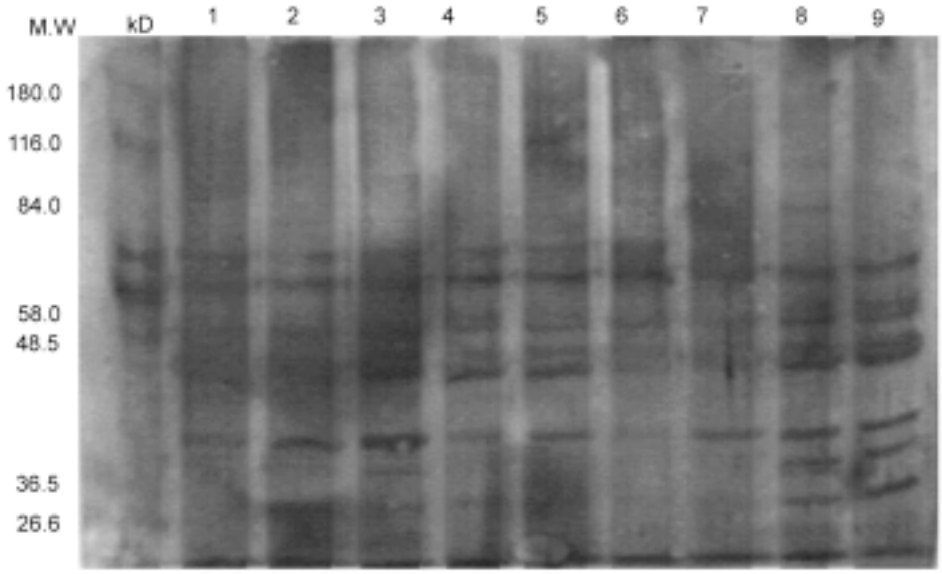

Fig. 1. Western-blot of nine isolated *Lactococus garvieae* strains (see Table 1).

fish, i.e., 6% mortality after 30 days compared to 54% mortality for non-immunized fish (88.8% RPS).

Discussion

In our study, the antigenic profiles of *L. garvieae* isolated from Turkey did not differ from those of isolates from Spain or England. However, the nine strains in our study produced six protein bands while Eldar et al. (1997) detected only one band of 48 kDa mw when a blot was developed with sera drawn from trout vaccinated against *Streptococcus iniae*. Barnes et al. (2002) suggested that the predominant immunoglobulin (Ig) binding proteins are similar among capsulated and non-capsulated isolates. The predominant Ig binding protein in all our isolates was approximately 52 kDa mw. In capsulated isolates, an additional strongly binding Ig band appeared at 30 kDa mw.

Elliot et al. (1991) identified their isolates as *L. lactis* subsp *lactis* and distinguished their isolates from *L. garvieae* by differences in protein bands in the 29-66 kDa range. *Lactococcus garvieae* had a major protein band around 43 kDa whereas *L. lactis* lacked this band. The protein bands in our work ranged 30-66 kDa, similar to those of Elliot et al. (1991). We determined 30-52 kDa bands in our isolates, similar to the *L. garvieae* isolates of Barnes et al. (2002).

Among other factors that impact the effectiveness of fish vaccines, the route of administration substantially affects the degree of protection conferred in *in vivo* experiments (Ellis, 1988; Newman, 1993) by affecting the efficiency of immunogenic constituent transfer of the vaccine to important recognition centers of the fish immune system. We obtained an RPS of 88.8% 30 days after intraperitoneal administration of the prepared bacterin. Eldar et al. (1997) obtained an RPS of 90% against *S. iniae* and Romalde et al. (2004) an RPS of 83.3% against *L. garvieae* after intraperitoneal immunization of fish, similar to our results.

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