Short communication

Safety and efficacy of an inactivated vaccine against *Lactococcus garvieae* in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

We studied the safety and efficacy of an inactivated vaccine (Ichtiovac-Lg) against *Lactococcus garvieae* in rainbow trout (*Oncorhynchus mykiss*). In an initial dose-response experiment to test safety, we injected 50 rainbow trout weighing 30–40 g with a double dose of vaccine (0.2 ml) intraperitoneally. We observed these fish three times a day until day 50 post-vaccination when they were killed to evaluate visceral reactions, adhesions and intraperitoneal absorption. Survival was 100% in both the treatment and control groups and no significant differences were found in percentage of severe adhesions and pigmentation of peritonea and viscera.

In a second trial, we injected 50 rainbow trout weighing 30–40 g with 0.1 ml of vaccine and a control group was injected with 0.1 ml of PBS intraperitoneally. On day 29 post-vaccination, both groups were challenged by intraperitoneal injection with 0.1 ml of a virulent heterologous strain of *L. garvieae* at 3×10^6 cfu ml^{-1} and fish were observed for a further 21 days. At the end of the experiment, the survivals of the vaccinated fish and control group were 94 and 4%, respectively.
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1. Introduction

Lactococcosis caused by *Lactococcus garvieae* is an important infectious disease of farmed rainbow trout (*Oncorhynchus mykiss*) in many countries when water temperature rises above 16 °C in summer months. *L. garvieae* is a Gram-positive, non-motile, ovoid cocci, occurrs in pairs and short chains, produces α-haemolytic colonies on blood agar, and is oxidase and catalase negative, non-acid fast, and non-sporulating (Ravelo et al., 2001; Vendrell et al., 2006). This pathogen has been isolated in marine and freshwater aquaculture; it has been reported with increasing frequency in the Mediterranean area (Ceschia et al., 1992; Eldar and Ghittino, 1999; Vela et al., 2000; Pereira et al., 2004). The clinical manifestations of this agent in trout are characterized by uni- or bilateral exophthalmia with haemorrhage in the periocular area, in the opercula, in the buccal area, at the base of the fins, and on the surface, darkening of the skin, and distended abdomen. Internally, the peritoneal cavity can also present haemorrhage and purulent exudates (Doménech et al., 1993; Afonso et al., 2003).

Lactococcosis can cause important economic losses that need the use of chemical treatments, especially antibacterials (Lewin, 1992). However, the improper use of antibacterials can cause antibiotic resistance, legal restrictions and difficulties due to anorexia. Therefore, a safe and efficacious vaccine is needed.

Vaccines containing oil adjuvants have two advantages: longer-lasting protection and elevated titers of protective antibodies.

Our aim was to investigate the safety and efficacy of an inactivated vaccine (Ichtiovac-Lg, Hipra, S.A. laboratories) emulsified with oily adjuvants (Aquamun) against an experimental infection with a heterologous strain of *L. garvieae* in rainbow trout (*O. mykiss*).

2. Material and methods

2.1. Bacterial strain

We used a virulent capsulated strain of *L. garvieae* CLFP LG1 (Culture collection, Laboratory of Fish Pathology, University of Zaragoza, Spain) isolated from diseased rainbow trout in Spain. Inocula of *L. garvieae* were prepared by growing cells overnight on blood agar (BioMerieux, France) at 22 °C for 24 h, washing twice in sterile phosphate-buffered saline (PBS) and re-suspending to a density of 3×10^6 colony forming units (CFU) ml^{-1} in PBS. Cultures were checked for purity and CFU estimated by plate counting.

2.2. Experimental animals

Rainbow trout, average weight 35 ± 5 g (mean $\pm$ S.D.), were obtained from a commercial fish farm from the Autonomous Community of Aragon, Spain. The health status was examined immediately using conventional microbiological techniques upon arrival in the aquaria.

The trout were distributed in 1000-l tanks in fresh water, had a 25% water exchange every day and continuous aeration. They were stocked at a density of 50 fish per tank. To avoid biased divisions of fish in the different experimental groups, the 200 fish were co-mingling ahead of time and we scooped fish out in alternating fashion (first to tank 1, then to tank 2–4, and then back to the first, etc., with 5 fish each time up to 50 per tank). Fish were fed with a commercial pelleted diet (2% body weight/day). The water temperature was increased progressively from 15 ± 1 to 19 ± 1 °C to begin the safety and challenge tests. This temperature is suitable for multiplication of the agent and development of the disease.

2.3. Safety test

For this assay, we used the protocol established by Council Directive 81/852/EEC for the production and control of live and inactivated vaccines intended for fish (European Commission, 1994). The sample size was determined using Win Episcope 2.0 (Thrusfield et al., 2001) to establish a minimum difference of 25% between the variables (45 fish for a confidence level of 95% and a power of 80%). However, we added 10% to the original sample size to allow for possible loss of sample. Thus, 50 fish were injected intraperitoneally with a volume of vaccine corresponding to twice the recommended dose per unit mass (0.2 ml) and the control group was injected with 0.2 ml of PBS. The trout were observed three times a day for an abnormal local or systemic reaction, until day-50 post-vaccination when all fish were killed to evaluate visceral reactions, adhesions and intraperitoneal absorption. All fish were euthanized by immersion in a tank containing tricaine methanesulfonate (MS-222, Syndel Laboratories Ltd., Canada) at a concentration of 150 mg l^{-1} of water for 15 min. To evaluate weight gain, every fish was anaesthetised with a lower dose of MS-222 (50 mg l^{-1}) and weighed (MM-600, Gram Precision, Spain; precision 0.1 g, max. 600 g) at the start and end of the assay. The study was approved by the Zaragoza University ethics committee.

2.4. Challenge test

The assay was designed according to specific requirements established by Council Directive 81/852/EEC for the production and control of fish vaccines (European Commission, 1994) that establish at least 50 fish should be vaccinated. Thus, 50 rainbow trout were vaccinated intraperitoneally with 0.1 ml of vaccine (dose recommended by manufacturer) and the control group was injected with 0.1 ml of PBS. On day-29 post-vaccination, the vaccinated and control groups were anesthetized and then challenged by intraperitoneal injection with 0.1 ml of a virulent heterologous strain of *L. garvieae* at 1×10^6 CFU ml^{-1} and the fish were maintained and observed during the following 21 days. During the subsequent disease outbreaks, mortalities were recorded daily until the completion of experiment. The cause of death and pathological signs were verified by isolation and purification of bacteria from tissue samples of freshly dead fish and survivors on blood-agar plates and bile-esculin-agar plates (Difco, USA) at 22 °C for 72 h and identification by a previously described PCR method (Zlotkin et al., 1998). In addition, cumulative mortality was recorded and the vaccine efficacy was calculated on the last day

of the trial by relative percent survival (RPS; Amend, 1981).

$$RPS = \left(1 - \frac{\% \text{ mortality in vaccinated group}}{\% \text{ mortality in control group}}\right) \times 100$$

2.5. PCR conditions for L. garvieae identification

Bacterial cultures (1.0 ml) and spleen, kidney, liver, and encephalon samples (250 mg) were diluted in 200 µl of Tris–EDTA buffer (pH 8.0; 10 mM Tris, 1mM EDTA), and centrifuged at $12,000 \times g$ for 1 min, and pellets were extracted with InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) in accordance with manufacturer's protocol. The amplification of the extracted DNA was carried out by *L. garvieae* 16S rDNA gene specific primers, pLG-1 (5′-CATAAC AATGAG AATCGC- 3′) and pLG-2 (5′-GCACC CTCGC GGGTTG-3′) with a GeneAmp PCR System 2400 termal cycler (Perkin-Elmer, USA). The amplification steps include initial denaturation at 94 °C for 5 min, followed by 35 cycles of each consisting of a denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 45 s plus final extension for 5 min at 72 °C after 35 cycles. The amplified product was maintained at 4 °C for 5 min. The PCR-amplified samples were subjected to electrophoresis (90 min, 90 v) in 2% agarose gel (Bio-Rad Laboratories, USA) with $1 \times$ TBE buffer (40 mM Tris–borate, 1 mM EDTA [pH 8]) and stained with ethidium bromide. The DNA molecular-weight marker was a 100-bp ladder (Bio-Rad Laboratories) to estimate amplified fragment size (Zlotkin et al., 1998). To establish the sensitivity of the PCR assay for detecting *L. garvieae*, we used 10-fold serial dilutions of pure culture that were simultaneously plated to calculate the number of colony forming units.

2.6. Statistical analysis of data

Cumulative survival of vaccinated and unvaccinated groups was subjected to Pearson's chi-square test. Survival analysis of the challenge test was calculated using the Kaplan–Meier method and compared by the log-rank test. All probability values were two-sided confidence intervals (CI) and a value of $P = 0.05$ was considered significant. Data were analysed using SPSS for Windows version 11.5 (SPSS, Chicago, IL). Prevalence of each lesion or effect was performed using EpiCalc 2000, Version 1.02 (Gilman and Myatt, 1998).

3. Results

3.1. Safety test

Survival was 100% in both groups. The severity of the side effects observed during necropsy was classified according to European guideline for the use of adjuvanted veterinary vaccines (European Agency for the Evaluation of Medicinal Products, CVMP/ IWP/043/97).

Table 1
Classification and prevalence of the side-effects observed in both vaccinated and control groups during the safety test of an inactivated vaccine against *L. garvieae* in rainbow trout

Side-effects	Vaccinated fish ($n = 50$)		Control ($n = 50$)
	Prevalence (%)	95% CI	Prevalence (%)
External abnormal appearance[a]	0	0, 9	0
Moderate adhesions between viscera[b]	56	41, 70	0
Major adhesions between viscera[c]	6	2, 18	0
Moderate adhesions between viscera and abdominal wall[d]	30	18, 45	0
Major adhesions between viscera and abdominal wall[e]	3	0.4, 13	0
Sparse pigmentation of the visceral peritoneum[f]	8	3, 20	0
Massive pigmentation and opacity of peritoneum[g]	0	0, 9	0
Laceration of peritoneum after evisceration[h]	0	0, 9	0
Vaccine remains[i]	26	15, 41	0

[a] Clinical symptoms of ill (exophthalmia, haemorrhage in the periocular area, opercula, buccal area or at the base of the fins, darkening of the skin, distended abdomen).

[b] Slight adhesions between abdominal organs seen as tiny fibrous tissue, the adhesions are easily detached and organs are intact following detachment.

[c] Firm adhesions of fibrous connective tissue connecting greater parts of the abdominal organs that give the viscera a "one-unit" appearance.

[d] Localised adhesions between viscera and abdominal wall, most frequently seen close to the injection site, that do not cause damage in the peritoneum when are separated.

[e] Extensive adhesions between abdominal organs and abdominal wall without damage to the peritoneum when are separated.

[f] Slight opaqueness or sparse pigmentation in localised areas of the peritoneum.

[g] Obvious thickening and opaqueness of greater areas of the peritoneum.

[h] Severe damage in the abdominal wall (peritoneum and muscle) when viscera are removed following evisceration.

[i] Small drops of vaccine remaining in the abdominal cavity.

Moderate adhesions, most frequently localised close to the injection site, minor pigmentation of the visceral peritoneum and moderate adhesions between viscera were visible in the vaccinated group (Table 1). However, these results are considered acceptable by the European Pharmacopoeia (2002).

The starting weights were 34 ± 4 g for the vaccinated group and 35 ± 4 g for the control group, and the weights at termination of the experiment were 54 ± 6 and 59 ± 6 g, respectively. Reduced appetite was observed in the vaccinated group for approximately 2 weeks after vaccination. The fish on the control group returned to feed faster (they were taken feed 12 h after intraperitoneal injection) than the vaccinated group. Fish were offered the same volume of pellets than previous injection but in vaccinated group a part of the feed left in the floor of the tank (data not recorded). Vaccinated fish returned to feed to the same level as controls progressively in the following 13 days after vaccination.

3.2. Efficacy of the vaccine

In the control group, fish began to die on the third day post-injection. Fish presented the typical signs of the disease, with a rapid anorexia, uni- or bilateral exophthalmia, melanosis, abdomen distension, anal prolapses and haemorrhaging at the base of the fins.
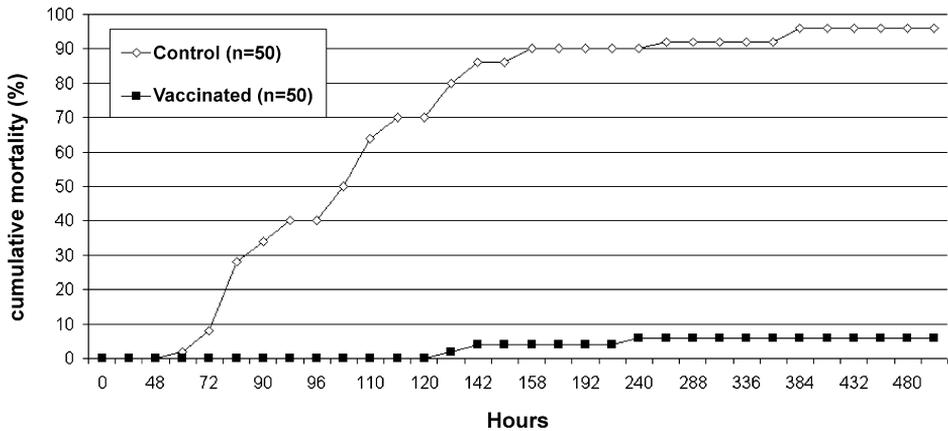
Fig. 1. Mortality of rainbow trout during an infection-challenge experiment initiated 1 month after intraperitoneal vaccination against *L. garvieae*.

The cumulative survivals of the vaccinated and control fish were 94% (95% CI 87, 100%) and 4% (95% CI 0, 9%), respectively (log-rank test $P < 0.0001$; Fig. 1). The RPS was 94%; vaccines with an RPS value $> 70\%$ are considered acceptable (European Pharmacopoeia, 2002).

3.3. Examination of tissues/organs during and after challenge test

Control fish that died during the study had typical lactococcosis lesions (congestion of all internal organs, haemorrhages in liver, ascitis with presence of abundant yellowish exudates and haemorrhagic enteritis). Survivors in the vaccinated group showed no pathological signs. However, the microbiological analysis of intestinal
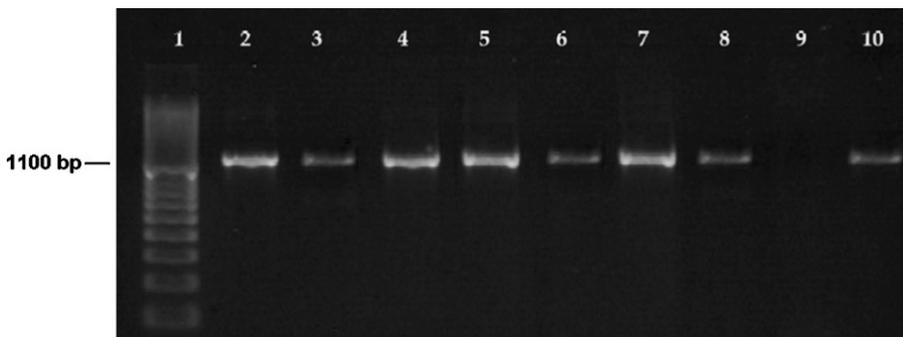

Fig. 2. Specificity of the *L. garvieae* PCR assay. Agarose gel (2%)-resolved ethidium bromide-stained PCR products from template DNA. Lanes: (1) DNA molecular-weight marker; (2) kidney tissue sample at 4 days post-infection; (3) kidney tissue sample at 16 days post-infection; (4) spleen at 4 days post-infection; (5) encephalon at 4 days post-infection; (6) encephalon at 16 days post-infection; (7) liver at 4 days days post-infection; (8) liver at 16 days days post-infection; (9) negative control; (10) positive control.

content from both unvaccinated and vaccinated survivors showed the presence of *L. garvieae*.

L. garvieae was a dominant bacterium isolated from spleen, kidney, liver, and encephalon samples of unvaccinated survivors. The bacterium was also isolated from freshly dead fish. The PCR assay resulted in the amplification of a band of 1100 bp that was detected for all samples tested from unvaccinated group (Fig. 2). These data confirmed the presence of *L. garvieae*. In addition, the PCR assay demonstrated a detection limit of 8 CFU for *L. garvieae* when pure cultures were used.

4. Discussion

We suspect that some of the efficacy was due to the inclusion of the mineral-oil adjuvant. The immuno-enhancing effect of this kind of adjuvant has been demonstrated in Atlantic salmon (*Salmo salar* L.) vaccinated against furunculosis (Midtlyng et al., 1996).

We used fish with the minimum body mass recommended and they were injected with a volume of vaccine corresponding to twice the recommended dose per unit mass, and no mortality was observed. Moderate lesions observed during the safety test were considered acceptable for this kind of vaccine, because the intra-abdominal lesions consistently seen as side effects after intraperitoneal vaccination were deemed to be of minor importance. The vaccination did not have important negative effects on the weight of vaccinated fish (control group 59 ± 5 g and vaccinated group 54 ± 6 g), although reduced appetite was seen for approximately 2 weeks after vaccination. Oil-adjuvant injections have previously been shown to cause intra-abdominal adhesions in vaccinated fish, such as halibut (Bowden et al., 2000; Gudmundsdóttir et al., 2003) and salmon (Poppe and Breck, 1997; Midtlyng and Lillehaug, 1998; Sørum and Damsgård, 2004).

Our results clearly demonstrate that under our experimental conditions the intraperitoneal vaccination using oil adjuvants in rainbow trout showed some moderate intra-abdominal lesions while major lesions showed lower prevalence, which are considered acceptable by European Pharmacopoeia. Intraperitoneal vaccination 1 month before intraperitoneal experimental challenge with *L. garvieae* was quite successful: the RPS was 94% (94% survival of the 50 vaccinated trout versus only 4% survival of the controls).

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