

Efficacy of single and combined *Streptococcus iniae* isolate vaccine administered by intraperitoneal and intramuscular routes in tilapia (*Oreochromis niloticus*)

Phillip H. Klesius^{a,*}, Craig A. Shoemaker^a, Joyce J. Evans^b

^a USDA, ARS Aquatic Animal Health Laboratory, P.O. Box 952, Auburn, AL 36830 USA

^b 300 Washington Avenue, Chestertown, MD 21620 USA

Received 14 October 1999; received in revised form 7 February 2000; accepted 13 February 2000

Abstract

We evaluated the effectiveness of *Streptococcus iniae* vaccines prepared from formalin-killed cells and concentrated extracellular products (greater than 2 kDa) of a single isolate of *S. iniae* (ARS-10) and a combination of ARS-10 + ARS-60 *S. iniae* isolates in tilapia, *Oreochromis niloticus*, for the prevention of streptococcal disease. Two routes of administration, intraperitoneal (i.p.) and intramuscular (i.m.) injection, were evaluated for vaccine efficacy. Tilapia were vaccinated by i.p. or i.m. injection of the vaccine or tryptic soy broth (control) and challenged by i.p. injection with 1×10^8 colony-forming units of *S. iniae* 30 days post-immunization. Tilapia i.p. immunized with ARS-10 vaccine and challenged with the homologous isolate (ARS-10) had a relative percent survival (RPS) of 45.6%. Tilapia i.p. immunized with ARS-10 vaccine and challenged with a heterologous isolate (ARS-60) had an RPS of 93.7%. In contrast, we found that the RPS was 17.7% in tilapia i.m. immunized with the ARS-10 vaccine and challenged with the same isolate, ARS-10. Heterologous (ARS-60) isolate challenge resulted in an RPS of 59.5%. However, the i.m. administration of a vaccine combining the ARS-10 + ARS-60 isolates provided an RPS of 63.1 against ARS-10 isolate and RPS of 87.3% against ARS-60 isolate in comparison to RPS of 17.7 and 59.5 against ARS-10 and -60 isolates provided by the single isolate ARS-10 vaccine. Vaccination significantly reduced abnormal behavior and morphology. We found a highly significant ($P < 0.01$) negative correlation between the behavioral and morphological score and

* Corresponding author. Tel.: +1-334-887-3741; fax: +1-334-887-2983.
E-mail address: klesiph@vetmed.auburn.edu (P.H. Klesius).

RPS. Serologic evaluation revealed that both i.p. and i.m. immunizations stimulated significant ($P < 0.05$) antibody titers in comparison to the non-immunized tilapia. Significantly ($P < 0.05$) increased antibody titers were also produced by i.m. immunization of the combined ARS-10 + ARS-60 vaccine. Administration of the single isolate ARS-10 vaccine by i.m. injection was marginally protective in preventing streptococcal disease caused by homologous and heterologous isolates of *S. iniae*. The variability seen in the protective ability of a single *S. iniae* isolate indicates that antigenic heterogeneity exist in *S. iniae*. Recognition of this antigenic variability is very important to development of efficacious streptococcal vaccines. Thus, this research suggests that efficacious vaccines, that may be administered by either i.p. or i.m. injection, are dependent on the antigenic composition of the *S. iniae*. Published by Elsevier Science B.V.

Keywords: Vaccines; *Streptococcus iniae*; Routes of immunization; Antibody titers

1. Introduction

Tilapia (*Oreochromis niloticus*) production is subjected to heavy economic losses due to mortality caused by *Streptococcus iniae* (Shoemaker and Klesius, 1997). Antibiotic treatment is ineffective and the need for a vaccine is paramount in the control of streptococcal disease. Rainbow trout (*Oncorhynchus mykiss*) intraperitoneally (i.p.) immunized with formalin-killed *Streptococcus* sp. in Freund's incomplete adjuvant were protected against *Streptococcus* sp., whereas trout immunized by bath immersion were not protected (Akhlaghi et al., 1996). Protection of rainbow trout after i.p. vaccination with killed *S. iniae* vaccine was recently reported (Eldar et al., 1997). This formalin-killed *S. iniae* vaccine protected trout in both experimental and field conditions for up to 4 months. An *S. difficile* formalin-killed vaccine was reported to protect tilapia against challenge with *S. difficile* (Eldar et al., 1995; Bercovier et al., 1997). Turbot (*Scophthalmus maximus*) were protected against *Enterococcus* sp. after vaccination with a toxoid-enriched bacterin (Toranzo et al., 1995; Romalde et al., 1996). The toxoid-enriched bacterin vaccine was a combination of two formalin-killed *Enterococcus* sp. isolates and their culture fluids. Recently, we reported the efficacy of killed *S. iniae* vaccine in tilapia (Klesius et al., 1999). The objective of this study was to further evaluate the efficacy of *S. iniae* vaccines prepared from formalin-killed cells and concentrated extracellular products (greater than 2 kDa) using a single isolate (ARS-10) and combination of two isolates of *S. iniae* (ARS-10 and ARS-60) in tilapia, *O. niloticus*, for the prevention of streptococcal disease. The efficacy of the single and combined isolate vaccines were evaluated by homologous and heterologous isolates challenge after i.p. or i.m. administration.

2. Materials and methods

2.1. Tilapia

The tilapia were from stocks maintained at the ARS, USDA, Aquatic Animal Health Research Laboratory (Auburn, AL). Tilapia (mean weight of 18 ± 2 g) were acclimated

in flow-through 57-l glass aquaria supplied with 0.5 l/h dechlorinated water for 10 days prior to experiments. Five replicate tanks of 25 fish each were used in all immunization experiments. A light and dark period of 12:12 h was maintained and aeration was supplied by an air stone. The fish were fed daily to satiation with Aquamax Grower 400 (Brentwood, MO). To verify the *S. iniae*-free status of the fish, samples were obtained for bacterial culture by passing an inoculation loop into brain and kidney. The samples were streaked directly on sheep blood agar that were incubated at 27°C for 24 to 48 h. *S. iniae* was not isolated from five randomly selected fish.

2.2. Water quality

The dissolved oxygen, temperature and salinity were measured daily using a YSI 85 oxygen conductivity, salinity, and temperature meter (Yellow Spring Instrument, Yellow Springs, OH). The pH, hardness, ammonia and nitrites were determined daily using the Fresh Water Aquaculture Kit Model AG-2 (LaMotte, Chestertown, MD). In all trials, the mean \pm standard deviation of dissolved oxygen (mg/l) was 6.02 ± 0.561 , temperature (°C) was 25.4 ± 0.627 , pH was 7.2 ± 0.0 , salinity (%) was 0.1 ± 0.00 and hardness was 110 ± 10.0 . Ammonia and nitrite concentrations (mg/l) were 0.

2.3. Bacteria

S. iniae isolates ARS-10 originally isolated from tilapia and ARS-60 isolated from hybrid striped bass with natural streptococcal disease were used to infect fish. The isolates were identified as *S. iniae* by standard methods (Shoemaker and Klesius, 1997). Isolates were grown in tryptic soy broth (TSB, Difco Laboratories, Sparks, MD) for 24 h at 27°C and then were frozen in 0.2-ml aliquots at -70°C . The infectious isolates used in this study were prepared by inoculating 250 ml of TSB in 500-ml culture flask with a thawed aliquot of the frozen isolate. The cultures were adjusted to an optical density of 1.2 at 540 nm using a spectrophotometer to give a *S. iniae* concentration of 1×10^8 colony forming units (CFU) /ml (determined by previous plate count) after 24 h at 27°C incubation. Fish were then challenged by i.p. injection of 100 μl *S. iniae*.

2.4. Vaccine preparation

Vaccines were prepared similar to Klesius et al. (1999). Briefly, *S. iniae* isolates (ARS-10 and ARS-60) were separately cultured in TSB and incubated in a shaker (70 RPM) water bath at 27°C for 72 h. Cultures were treated with 10% neutral buffered formalin to give a final concentration of 3% at 27°C for 24 h. The formalin-treated cultures were centrifuged at $7000 \times g$ for 30 min and cell pellet and culture fluid separated. The cell free culture fluid was concentrated 20-fold using a 2-kDa hollow fiber concentrator, filter-sterilized (0.2 μm) and used to re-suspend the cell pellet at V/V of 10:1. The final concentration of the vaccines were 4×10^9 CFU/ml (determined by previous plate count) or 1.9 optical density at 540 nm. The combined vaccine was produced using equal volumes of each vaccine ARS-10 and ARS-60. Non-vaccinates received concentrated TSB only. The vaccines were determined to be killed by lack of growth on sheep blood agar at 72 h.

2.5. Vaccination protocol

The ARS-10 or the combined ARS-10 + ARS-60 vaccines were either injected i.p. or i.m. in a volume of 0.1 ml into tilapia. Control tilapia received 0.1 ml of TSB by either route. Immunized and control tilapia were held for 30 days before challenge. The tilapia were monitored for mortality for 14 days post-challenge (Klesius et al. 1999).

2.6. Bacteriologic sample collection and evaluation

Dead fish were removed twice a day and at post-mortem examination, specimens were obtained aseptically from brain and kidney sites. Specimens were cultured directly onto sheep blood agar at 27°C for 24–48 h. Beta-hemolytic, catalase-negative and Gram-stained positive coccus colonies were subcultured onto sheep blood agar and then bacteriologically and biochemically identified as *S. iniae* according to tests described by Shoemaker and Klesius (1997). All tests were conducted at 27°C using media purchased from Remel (Lenexa, KS).

2.7. Mortality, behavioral and morphological score

The data were recorded on a daily behavioral and morphological checklist that included mortality, location of fish in the aquarium, swimming pattern, feeding response, activity/excitability and morphology. For the purpose of this study, one or more fish in an aquarium exhibiting behavioral and morphological signs of *S. iniae* were graded according to the following system:

0: Normal behavior or morphology.

1: Surface or bottom location; any form of erratic swimming; slow acceptance of food; hyperactive, lethargic or unresponsive; and darkened skin coloration.

2: No acceptance of food or eye opacity or body curvature.

The accumulative score was calculated as the mean score of five aquaria per treatment at 14 days post-challenge. The efficacy of the vaccine was calculated as the relative percent survival (RPS) according to Amend (1981).

2.8. Blood collection and serologic evaluation

The blood sample was collected by venipuncture, placed into a micro-centrifuge tube and allowed to clot for 1 h at 25°C and then centrifuged at $1000 \times g$ for 10 min. Serum was collected by Pasteur pipette and stored in a plastic-capped tube at -70°C until assayed for antibody titer.

Antibody titer against *S. iniae* in serum was determined by use of a microtitration agglutination test. Briefly, each well of a 96-round well microtitration plate was plated with 50 μl of phosphate-buffered saline (PBS) solution (pH 7.2–7.4) and then 50 μl of tilapia serum was added to the first well of each row, mixed and then 50 μl of diluted serum was serially diluted into the remaining wells. Doubling dilutions of positive and

negative sera were included on every plate as controls. To each well, 50 μ l of *S. iniae* cells (ARS-60) suspension was added and mixed. The plate was covered and incubated in humidified air at 25°C for 18 h. The highest serum dilution that showed a circular diffuse button with fuzzy edges at the bottom of the well was considered a positive reaction and a circular compact cell button was considered to be a negative reaction. Antibody titer was expressed as log base₁₀ for each isolate studied.

2.9. Statistical analysis

Mortality and behavioral and morphological data were analyzed by one way analysis of variance using Duncan's multiple-range test (SAS Institute, 1997). RPS and antibody titer values were analyzed by Pearson correlation coefficient (SAS Institute, 1997). Significant differences were determined at $P < 0.05$ and $P < 0.01$.

3. Results

Tilapia i.p. immunized and challenged with the homologous *S. iniae* ARS-10 isolate had a mean percent mortality of 34.4 and RPS of 45.6 (Table 1). In contrast, the mean percent mortality was 52.0 and the RPS was 17.7 by the i.m. route of immunization (Table 1). Heterologous isolate challenge with the ARS-60 isolate of tilapia i.p. immunized resulted in a RPS of 93.7. Tilapia i.m. immunized and then challenged with the heterologous *S. iniae* ARS-60 isolate had a RPS of 59.5. Statistically, there was a significant ($P < 0.05$) difference in the mean percent mortality between i.p. and i.m. routes of immunization. The ARS-10 vaccine administered only by i.p. route provided significant protection against both the homologous and heterologous *S. iniae* isolates (Fig. 1). Mortality began at 4 days post-challenge in the i.p. and i.m. ARS-10 vaccinates and the non-vaccinates control fish, however the i.p. vaccinates had significantly ($P < 0.05$) less mortality than the control fish at 14 days post-challenge. Fish vaccinated by the i.m. route showed significantly decreased mortality only with ARS-60 isolate challenge at 14 days. However, i.m. immunization of tilapia with a combined ARS-10 + ARS-60 vaccine had RPS of 63.1 against *S. iniae* ARS-10 and 87.3 against *S. iniae* ARS-60 isolates (Table 1). The accumulative mortality in the vaccinates and non-vaccinates is shown in Fig. 2. The combined vaccine provided significant protection against the ARS-10 isolate where monovalent ARS-10 vaccine provided none. The mean percent mortality of the non-vaccinates was 63.2%.

Highly significant differences ($P < 0.01$) were noted between the accumulative behavioral and morphological scores of the vaccinates and non-vaccinates (Table 2). The vaccinates had a 70.9% reduction in their mean score. A highly significant difference was also noted between vaccinates administered the ARS-10 and route of immunization. The behavioral and morphological score for i.p. vaccinates was 4.4 in comparison to a score of 12.4 for i.m. vaccinates. There was a highly significant negative correlation between the behavioral and morphological score and RPS ($r = -0.99$, $P = 0.0003$). The behavioral and morphological signs observed in the control non-vaccinates included surface or bottom erratic swimming, refusal of food, lethargic activity, darkening skin

Table 1

Mortality and antibody titer of *S. iniae* vaccinated and non-vaccinated tilapia after challenge with *S. iniae*

Accumulative mortality at 14 days post-challenge or 44 days post-immunization. Tilapia with an average weight of 18 ± 2 g were stocked at 25 fish per tank into five replicate tanks for each treatment, respectively.

<i>S. iniae</i> vaccine	Route	Challenge isolate	Accumulative mortality	Mean % mortality (S.E.M.)	RPS	<i>S. iniae</i> titer (mean log base ₁₀) ¹
ARS-10	i.p.	ARS-10	43	34.4 (± 0.7) ^c	45.6	–2.8896 ^c
ARS-10	i.p.	ARS-60	4	4.0 (± 0.7) ^c	93.7	–3.0702 ^c
ARS-10	i.m.	ARS-10	65	52.0 (± 1.3) ^a	17.7	–2.6087 ^{de}
ARS-10	i.m.	ARS-60	32	25.6 (± 1.0) ^d	59.5	–2.7893 ^{de}
ARS-10 + ARS-60	i.m.	ARS-10	29	23.3 (± 1.8) ^b	63.1	–2.2337 ^{cd}
ARS-10 + ARS-60	i.m.	ARS-60	10	8.0 (± 0.7) ^e	87.3	–2.0067 ^{bc}
TSB ²	Control	ARS-10/ARS-60	79	63.2 (± 1.4) ^a		–1.2441 ^a

¹Mean serum antibody titer at 14 days post-challenge and different superscripts indicate significant differences at the 95% level.

²Non-vaccinates received tryptic soy broth (TSB) only.

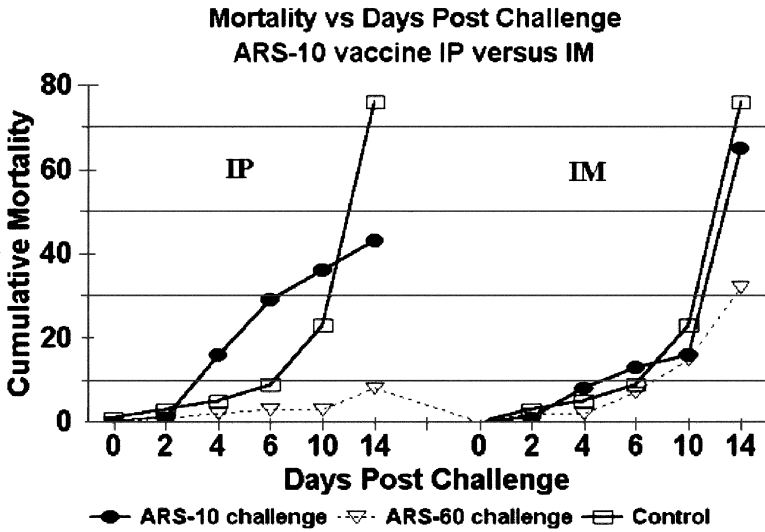


Fig. 1. Effect of i.p. and i.m. vaccination with the ARS-10 *S. iniae* vaccine on accumulative mortality due to *S. iniae* challenge in tilapia.

coloration and eye opacity. Vaccinates exhibited normal feeding and less frequent signs of abnormal behavior and morphology.

Statistical analysis of anti-streptococcal titer revealed significant differences ($P < 0.05$) between vaccinates and non-vaccinates (Table 1). No significant ($P > 0.05$)

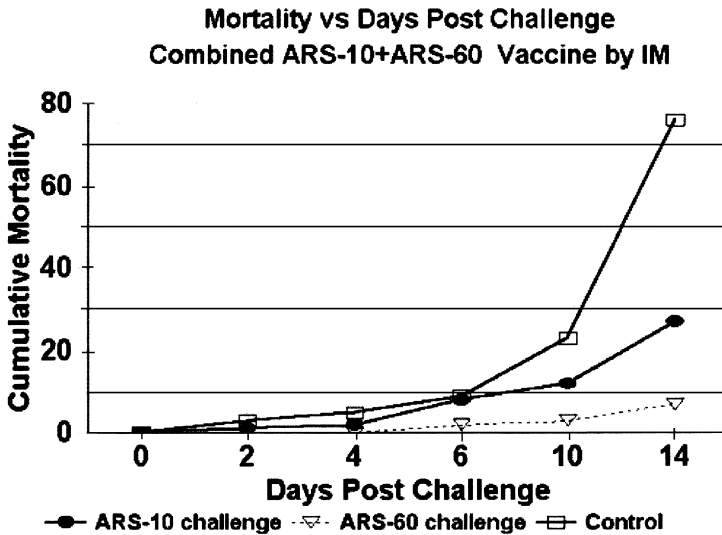


Fig. 2. Effect of i.m. vaccination with the combined ARS-10 and ARS-60 *S. iniae* vaccine on accumulative mortality due to *S. iniae* challenge in tilapia.

Table 2

Accumulative behavioral and morphological scores in vaccinates and non-vaccinates challenged with *S. iniae*. Mean accumulative behavioral and morphological score at 14 days post-challenge or 44 days post-immunization. Tilapia with an average weight of 18 ± 2 g were stocked at 25 fish per tank into five replicate tanks for each treatment, respectively.

<i>S. iniae</i> vaccine	Route	Challenge isolate	Mean accumulative score ¹
ARS-10	i.p.	ARS-10	9.2 ^{bcd}
ARS-10	i.p.	ARS-60	4.4 ^d
ARS-10	i.m.	ARS-10	12.4 ^b
ARS-10	i.m.	ARS-60	8.2 ^{bcd}
ARS-10 + ARS-60	i.m.	ARS-10	8.8 ^{bcd}
ARS-10 + ARS-60	i.m.	ARS-60	5.6 ^{cd}
TSB ²	Control	ARS-10/ARS-60	27.8 ^a

¹Different superscripts indicate significant differences at the 95% level.

²Non-vaccinates received tryptic soy broth (TSB) only.

differences in anti-streptococcal titers were noted in the vaccinates between the routes of immunization and *S. iniae* isolates used for challenge. The ARS-10 + ARS-60 combined vaccine stimulated slightly lower titers than did the single ARS-10 isolate vaccine. The antibody titer and RPS values among the i.p. and i.m. vaccinates did not correlate ($r = 0.09$).

4. Discussion

Vaccination with *S. iniae* vaccine by i.p. injection resulted in acquired immunity against infection with homologous and heterologous *S. iniae* isolates. The i.p. injection of the single isolate vaccine produced RPS of 45.6 and 93.7 against homologous and heterologous *S. iniae* isolates, respectively. However, differences exist between i.p. and i.m. routes of administration in their ability to induce protective immunity against the homologous and heterologous isolates. The results of i.m. injection were RPS of 17.7 and 59.5 against the homologous and heterologous *S. iniae* isolates, respectively. The basis for this difference is unknown, but differences in antigens between these two isolates are suggested. Tilapia i.m. vaccinated with the combined vaccine of both isolates had significantly enhanced protection compared to the single isolate vaccine following challenge with the homologous and heterologous isolates. Vaccination significantly ($P < 0.05$) reduced abnormal behavior and morphology. Thus, the ability to prevent streptococcal disease by the *S. iniae* vaccine administered i.m. was significantly ($P < 0.05$) increased by combining the two *S. iniae* isolates.

In a previous study, we found that i.p. injection of the same single isolate *S. iniae* vaccine reduced mortality by 91.3% in tilapia weighing 25 and 100 g (Klesius et al., 1999). These vaccinates were challenged with the same homologous *S. iniae* isolate. In the present study, the tilapia weighed 18 g. The difference in efficacy in tilapia of 18 and 25 g against challenge with a homologous *S. iniae* isolate may indicate that younger tilapia are less efficient in producing the same degree of protective immunity against

both homologous and heterologous *S. iniae* isolates after immunization with a single isolate vaccine. Muzquiz et al. (1999) demonstrated pathogenicity of streptococcal disease in rainbow trout was age dependent. Our data suggest homologous vaccine efficacy may also be age dependent. However, the stimulation of younger tilapia with the combination vaccine significantly ($P < 0.05$) enhanced their capacity to produce protective immunity.

The literature indicated a lack of studies on streptococcal vaccines where protection against homologous and heterologous isolates of *Streptococcus* sp., especially *S. iniae* were experimentally investigated in tilapia or other farmed species of fish. Zlotkin et al. (1998) demonstrated that a single *S. iniae* clone infected gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*) and spine foot (*Siganus rivulatus*) by restriction fragment length polymorphism ribotyping. However, the antigenic heterogeneity of *S. iniae* isolates remains to be determined. The variability seen in the protective ability of a single *S. iniae* isolate indicates that antigenic heterogeneity exists and is important to development of efficacious streptococcal vaccines. Combined vaccines may produce superior products that may be administered by either i.p. or i.m. injection.

Antibody titers stimulated by i.p. injection of a single *S. iniae* vaccine were higher than those titers obtained by i.m. injection, although not significantly ($P > 0.05$) different. The combination vaccine administered i.m. stimulated significantly ($P < 0.05$) lower titers than the single *S. iniae* isolate vaccine by i.m. injection. Previous studies indicated that protective immunity against *S. iniae* was dependent on antibody against *S. iniae*. The results of the present study appeared to strengthen the role of antibody in protective immunity. However, we did not find a correlation between the antibody titer and RPS reflective of protective immunity. This research suggests that protective antibody response is dependent on the antigenic composition of the *S. iniae* used to prepare the *S. iniae* vaccine and a combined *S. iniae* vaccine may help overcome the antigenic heterogeneity of different *S. iniae* isolates. Further, i.p. administration of a single *S. iniae* vaccine gives superior efficacy in comparison to administration by the i.m. route.

References

- Akhlaghi, M., Munday, B., Whittington, R., 1996. Comparison of passive and active immunization of fish against streptococcosis (enterococcosis). *J. Fish Dis.* 19, 251–258.
- Amend, D.F., 1981. Potency testing of fish vaccines. *Dev. Biol. Stand.* 49, 447–454.
- Bercovier, H., Ghittino, C., Eldar, A., 1997. Immunization with bacterial antigens: infections with streptococci and related organisms. In: Gudding, R., Lillehug, A., Midtly, P.J., Brown, F. (Eds.), *Fish Vaccinology*. Karger, Basel, pp. 153–160.
- Eldar, A., Shapiro, O., Bejerano, Y., Bercovier, H., 1995. Vaccination with whole-cell vaccine and bacterial protein extract protects tilapia against *Streptococcus difficile* meningoencephalitis. *Vaccines* 13 (9), 867–870.
- Eldar, A., Horovitz, A., Bercovier, H., 1997. Development and efficacy of a vaccine against *Streptococcus iniae* infection in farmed rainbow trout. *Vet. Immunol. Immunopathol.* 56, 175–183.
- Klesius, P.H., Shoemaker, C.A., Evans, J.J., 1999. Efficacy of a killed *Streptococcus iniae* vaccine in tilapia (*Oreochromis niloticus*). *Bull. Eur. Ass. Fish Pathol.* 19 (1), 1–3.

- Muzquiz, J.L., Royo, F.M., Ortega, C., DeBlas, I., Ruiz, I., Alonso, J.L., 1999. Pathogenicity of streptococcosis in rainbow trout (*Oncorhynchus mykiss*): dependence on age of diseased fish. Bull. Eur. Ass. Fish Pathol. 19 (3), 114–119.
- Romalde, J.L., Silva, R., Riaza, A., Toranzo, A.E., 1996. Long-lasting protection against turbot streptococcosis obtained with a toxoid-enriched bacterin. Bull. Eur. Ass. Fish Pathol. 16, 169–171.
- SAS Institute, 1997. SAS User's Guide. SAS Institute, Cary, NC, version 6.12.
- Shoemaker, C., Klesius, P., 1997. Streptococcal disease problems and control — a review. In: Fitzsimmons, K. (Ed.), Tilapia Aquaculture 2 Northeast Regional Agricultural Engineering Service, Ithaca, NY, pp. 671–682.
- Toranzo, A., Devesa, S., Romalde, J., Lamas, J., Riaza, A., Leiro, L., Barja, J., 1995. Efficacy of intraperitoneal and immersion vaccination against *Enterococcus* sp. infection in turbot. Aquaculture 134, 17–27.
- Zlotkin, A., Hershko, H., Eldar, A., 1998. Possible transmission of *Streptococcus iniae* from wild fish to cultured marine fish. Appl. Environ. Microbiol. 64 (10), 4065–4067.