

## Vaccination of rainbow trout against *Streptococcus iniae* infection: comparison of different routes of administration and different vaccines

M. Soltani<sup>1\*</sup>; M. Alishahi<sup>1</sup>; S. Mirzargar<sup>1</sup> and Gh. Nikbakht<sup>2</sup>

msoltani@ut.ac.ir

- 1- Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, P.O. Box: 14155-6453 Tehran, Iran
- 2- Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, P.O. Box: 14155-6453 Tehran, Iran

Received: May 2006

Accepted: September 2006

**Abstract:** Antibody production and clinical efficacy (relative percent survival RPS) were measured in 40±5g rainbow trout after immunization with two types of *Streptococcus iniae* vaccines consisting of formalin killed cells (FKC) and FKC enriched with the bacterial extracellular products (ECP) administered by intraperitoneal (i.p), immersion and oral routes at 16±1°C for 18 weeks. No significant difference was found in antibody levels among the fish i.p immunized with FKC enriched with ECP plus Freund's adjuvant (FA), FKC plus FA and FKC vaccines ( $P>0.05$ ), whilst the antibody production was significantly higher in these three groups than fish immunized by immersion and oral routes of FKC and FKC enriched ECP ( $P<0.05$ ). Also, fish immunized by immersion route with FKC showed significantly higher antibody titer than fish orally vaccinated with FKC with or without ECP until 12 weeks post-immunization ( $P<0.05$ ). No significant difference was found in antibody titer between orally vaccinated fish and control groups ( $P>0.05$ ). The RPSs ranging 82.6-100, 73.9-95 and 73.9-91.7% were obtained in the fish intraperitoneally immunized with FKC enriched ECP plus FA, FKC plus FA and FKC vaccines, respectively, compared to 0% survival for the control fish. Also, RPS in fish vaccinated by the immersion route was in the range 45.8-30.4% after 18 weeks post-vaccination. Efficacy of oral vaccination of fish with FKC plus ECP was in range of 8.7-29% and that of fish orally vaccinated with FKC resulted in 8.7-20.8% protection.

**Keywords:** Vaccine, *Streptococcus iniae*, Streptococcosis, Rainbow trout

---

\* Corresponding author

## Introduction

The Gram positive *Streptococcus iniae* has been associated with outbreaks of disease in several commercial species of freshwater and marine fish including tilapia (*Oreochromis aureus* and *O. niloticus*), yellowtail (*Seriola quinqueradita*), hybrid striped bass (*Morone saxatilis*), turbot (*Scophthalmus maximus*) and rainbow trout (*Oncorhynchus mykiss*) in different regions (Toranzo *et al.*, 1995; Kusuda & Salati, 1999; Evans *et al.*, 2002; Chang *et al.*, 2002; Chen *et al.*, 2002; Klesius *et al.*, 2000; Soltani *et al.*, 2005).

Because of the ineffectiveness of antibiotic treatment, the need for a suitable vaccine to this economically important bacterial disease is paramount in the control of the disease outbreak (Klesius *et al.*, 2000; Shellby *et al.*, 2002).

A number of vaccines have been produced to control the disease outbreaks in the affected fish but most attentions have been paid to non-salmonid species (Iida *et al.*, 1981; Sakai *et al.*, 1989; Romalde & Toranzo, 2002; Klesius *et al.*, 1999, 2000; Evans *et al.*, 2004).

Concerning the salmonid species in particular rainbow trout minimum data is available at the present. Akhlaghi *et al.* (1996) evaluated the relative percent survival (RPS) of passively immunized rainbow trout challenged with a virulent strain of *Streptococcus* sp. later identified as *Lactococcus garveiae* (Schmidtke & Carson, 2003). The fish were intraperitoneally immunized with 0.1 ml/100g body weight of sheep, rabbit and trout fish anti-*Streptococcus* sp. Antibody. The RPSs of 88.8, 36 and 10% after 1 month; 33.3, 6.8 and 6.8% after 2 month; and 13.3, 0 and 6.6% after 3 months post-immunization were obtained, respectively. These authors also demonstrated RPS of 88.8 and 11.1% after 1 month, 38.1 and 4.7% after 2 month, and 36 and 0% after 3 months post-immunization of fish actively immunized with formalin-killed-cells of the bacterium as intraperitoneal and immersion routes, respectively. Eldar *et al.* (1997) reported rainbow trout weighing 30-50g intra-peritoneally immunized with a single and/or double doses of formalin killed *S. iniae* suspensions containing  $3 \times 10^{10}$ ,  $3 \times 10^{10}$  and  $3 \times 10^{10}$  CFU/fish at 16°C resulted in 90,

90 and 50% protection one month post-vaccination, respectively. These authors also demonstrated that fish i.p. immunized with a single dose of  $3 \times 10^{10}$  cells/fish reached in 90% survival up to 6 months post-immunization compared to 20-30% survival in unimmunized fish. They could also measure antibody titer of 1:20, 1:10 and 1:1 in immunized fish 1, 3 and 6 months post-vaccination, respectively. Also, these workers were able to demonstrate 80 and 60% protection in 50g rainbow trout passively immunized with rabbit and trout anti-*S. iniae* hyperimmune sera containing ferund adjuvant after 0 (simultaneously) and 4 days post-immunization, respectively.

The outbreak by *S. iniae* infection in farmed trout in different parts of Iran is now one of the trout industry obstacles because of unsuitable health management criteria such overcrowding and sanitary poor water quality particularly increasing of water temperature during summer period. This study was aimed to evaluate the and compare the efficacy of formalin killed cells (FKC) of the recovered *S. iniae* (Soltani *et al.*, 2005) with the toxoid enriched bacterin administered by intraperitoneal inoculation, immersion and oral routes.

## Materials and Methods

Rainbow trout weighing  $40 \pm 5$ g were obtained from a commercial trout farm with a history of no disease outbreak during the last 2 years. Fish were transferred into 2000 liter tanks with a well aerated constant water flow. Water quality parameters including water temperature, dissolved oxygen, hardness, pH, nitrite and ammonium were  $16 \pm 1^\circ\text{C}$ ,  $>8\text{mg/l}$ ,  $250\text{mg/l}$ , 7.8,  $<0.1\text{mg/l}$  and  $<0.01\text{mg/l}$ , respectively. Fish were adapted for 2 weeks prior to the experiment and were fed with commercial trout feed at 3% body weight three times a day.

Lyophilized ampoules of a master stock of *S. inane* previously recovered from a farmed trout in Iran (Soltani *et al.*, 2005) was used. Blood agar and triptic soya agar and/or broth containing 0.5% glucose were used as the culture media, and the bacterial cells were killed after incubation for 48 h at  $27^\circ\text{C}$  by adding formalin (3% V/V) for 24 hrs. at  $4^\circ\text{C}$ . The formalin treated cultures were centrifuged at  $7000 \times g$  for 25 min and



cell pellet and culture fluid separated. The formalin-killed-vaccine was adjusted by optical density to a final concentration of  $1 \times 10^{11}$  cells/ml in sterile phosphate buffer.

Formalin-killed cells (FKC) enriched with the bacterial extracellular products (ECP) was prepared as described by Klesius *et al.* (2000). Briefly, the cell free culture fluid was concentrated 20-fold using a 20 KDa hollow concentrator (Amicon filter), filter sterilized ( $0.2 \mu\text{m}$ ) and used to re-suspend the FKC pellet at V/V of 10:1. The vaccines (FKC, and FKC plus ECP) were determined to be killed by lack of growth on blood agar at  $25^\circ\text{C}$  for 72 hrs. The final concentrations of the vaccines by intraperitoneal, immersion and oral vaccination were  $1 \times 10^8$  cells/fish,  $1 \times 10^6$  cells/ml and  $1 \times 10^6$  cells/g food, respectively. When necessary, the enriched vaccine was mixed with complete Freund's adjuvant (FA) at 2:1. Also, the enriched vaccine was tested in 10 trout by intraperitoneal inoculation for the safety of toxic effect by ECP toxins and no side effect was observed.

The vaccination protocol is shown in Table 1. One hundred and fifty fish were used per every vaccination route. The enriched vaccine containing FA, FKC containing FA and FKC without FA were intraperitoneally injected in a volume of 0.1ml/fish. The FKC vaccine was applied as an immersion for 1 minute. Also, FKC vaccine and enriched vaccine were used as oral route by adding vaccine to daily feed for 14 days. Gelatin was used for coating the vaccine on the pellet. A number of 200 fish were vaccinated per every treatment. Also, a number of 200 fish were intraperitoneally injected with FA at 0.1ml/fish and 200 fish received 0.1ml/fish of sterile PBS and 200 fish were considered completely untreated. Prior to any manipulation, fish were anesthetized by immersion in a well-aerated bath containing 100mg/l clove oil.

The efficacy of different immunization regimes was determined by intraperitoneal inoculation of vaccinated and unvaccinated fish with the virulent strain of *S. iniae* at 3, 6, 9, 12.15 and 18 weeks post-immunization (24 fish per group per occasion) at the same water quality parameters previously mentioned. The applied dose was 0.1ml/fish equal to  $1.7 \times 10^7$  cells/fish corresponding in to  $\text{LD}_{50}$  96 hrs. as determined using a

lyophilized ampoule of *S. iniae* prior to the experiment. The mortality percent (mean) in each group was calculated. Relative percent (RPS),  $RPS = [1 - (\% \text{mortality in vaccinated fish} / \% \text{mortality in control fish}) \times 100]$  expressed as mean  $\pm$  standard error for two replicates was calculated from the mortalities in different groups and controls (Ellis, 1988).

Micro agglutination test was carried out in 96 well plates as described by (Roberson, 1990). The total volume employed in each reaction was 100  $\mu$ l. The plates were incubated overnight at room temperature. The appearance of a button with fuzzy edges at the bottom of the well was considered as a positive reaction, whereas the formation of a round precipitation with sharp contours was evaluated as a negative reaction.

The obtained data in each group were compared statistically with the other groups within an experiment using one way ANOV through SPSS software.

**Table 1: Vaccination protocol in rainbow trout weighing 40g against *S. iniae* at 16°C.**

Type of vaccine/antigen	Route of administration	Doses used
Enriched vaccine	Intraperitoneal	$1 \times 10^8$ cells/fish
(FKC+ECP+FA)	Intraperitoneal	$1 \times 10^8$ cells/fish
FKC+FA	Intraperitoneal	$1 \times 10^8$ cells/fish
FKC	Immersion	$1 \times 10^6$ cells/fish
FKC	Oral	$1 \times 10^6$ cells/fish
Enriched vaccine (FKC+ECP)	Oral	$1 \times 10^6$ cells/fish
FKC	Intraperitoneal	0.1ml/fish
FA	Intraperitoneal	0.1ml/fish
PBS		

## Results

Results of agglutination titer are shown in Fig. 1. Antibody titers were significantly higher in fish intraperitoneally vaccinated with enriched vaccine adjuvant, FKC plus adjuvant and FKC, respectively ( $P < 0.05$ ). However, no significant difference was found in antibody levels between these groups during the experiment ( $P > 0.05$ ). Also, fish immunized by immersion route with FKC vaccine showed significantly higher antibody titer than fish orally vaccinated with the enriched and FKC vaccines until 12 weeks post-immunization ( $P < 0.05$ ). No significant difference was found in antibody titer between orally vaccinated fish and control groups ( $P > 0.05$ ).

As shown in Fig. 2, fish intraperitoneally vaccinated with the enriched vaccine containing adjuvant, FKC containing adjuvant and FKC resulted in RPSs of 82.61-100, 73.91-95.05 and 73.91-91.67%, respectively, during 18 weeks post-vaccination. The highest protection for these vaccines was obtained 2-3 weeks post-immunization, while the lowest protection reached 18 weeks post-immunization. The RPS in fish vaccinated with immersion route was the highest (45.83%) and the lowest (30.43%) after 18 weeks post-vaccination. Efficacy of oral vaccination of fish vaccinated with the enriched vaccine was in range of 8.7-29.17% during 18 weeks post-immunization, while fish orally vaccinated with FKC resulted in 8.7-20.83% protection. Fish intraperitoneally immunized with only FA and sterile PBS had 8-21.74% and 0% survival throughout the experiment, respectively. Also, fish without any treatment gave 100% mortality during each challenge trail. The confirmation of mortality aetiology was performed by reisolation and characterization of *S. iniae* from the haematopoietic tissues, kidney and spleen, of the moribund/dead fish using standard microbiological procedures.

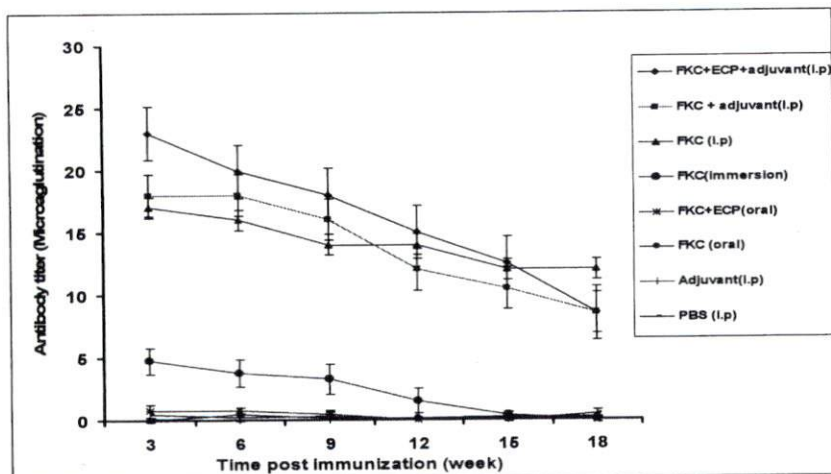


Figure 1: Microagglutination antibody titer (Mean±SE) of rainbow trout immunized against *S. iniae* with different vaccines and administered with different routes at 16°C. FKCECP=Formalin Killed Cells, ECP= Extra Cellular Products, IP=Intraperitoneal Injection.

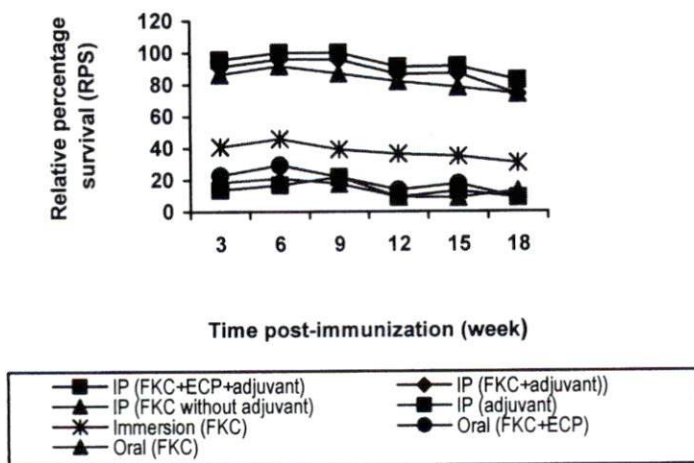


Figure 2: Relative percent survival (RPS) in rainbow trout immunized against *S. iniae* with different vaccines and different routes of administration at 16°C. FKCECP=Formalin Killed Cells, ECP= Extra Cellular Products, IP=Intraperitoneal Injection.



## Discussion

The results of survival of the immunized trout to the experimental disease along with the serological findings, agglutination titers, showed that the FKC vaccine with or without ECP elicited a protective immune response against *S. iniae*. The highest agglutinating titer found 1:23, was higher than reported by Eldar *et al.* (1997) who demonstrated a nil agglutinating titer in the intraperitoneally immunized trout with a protection of 50% survival after one month post-immunization with FKC of *S. iniae* at concentration of  $3 \times 10^8$  CFU/fish. Therefore, it could be concluded that any agglutinating titer in trout immunized to *S. iniae* is indicative protection against the disease as mentioned by Eldar *et al.* (1997). Also, in the preliminarily studies by Eldar *et al.* (1997), a protection of 90% was obtained in intraperitoneally immunized fish (only used 10 immunized fish per challenge) with higher dose of killed vaccine i.e.  $3 \times 10^{10}$  CFU/fish, for up to 6 month post-immunization compared to our RPS findings of 73.91-91.67% obtained after 18 weeks post-vaccination when trout were intraperitoneally immunized with a lower dose of  $1 \times 10^8$  cells/fish of FKC. Also, almost identical results (RPS=73.91-95.05) were obtained when fish were vaccinated with the FKC vaccine containing FA. However, the immunization of fish with FKC containing ECP resulted in a higher RPS (82.61-100) than the other routes of vaccination (Fig. 1) indicative of a positive efficacy due to the ECP in the vaccine. The analysis of the obtained qualitative data of humoral response to the vaccines showed that specific antibodies were directed to few proteins and probably against the glycocalix/capsular antigens. This was supported by the findings of Eldar *et al.* (1997) who obtained higher protection when high concentration of the FKC was used. Therefore, it seems that specific anticapsular antibodies are one of the important factors influencing the state of protection against *S. iniae* infection in trout. Further works are required to evaluate the possible role of capsular antigen in humoral response of fish against this Gram positive bacterial fish pathogen. Also, low antibody production verses high level of RPS findings in this study is indicative of probably an important role of the fish non-specific immune variables such as phagocytosis and lysozyme activity involved in protection against this disease. Further works require assessing the role of non-specific immunity of fish



to streptococcosis. This is particularly true when immunized fish are treated with immunostimulators, such as glucans, then significantly higher protection can be obtained (unpublished data).

Also, use of FA in FKC vaccine did not significantly enhance the rate of RPS or antibody titer. This is supported by the finding of very low levels of RPS and antibody titer in the fish intraperitoneally immunized with FA (Table 3). Therefore, it seems that use of FA in injectable vaccines is unnecessary, a fact that is of primary importance from the legal and economic point of view. Fish farmers whose fish production cycle is short (6 months) could benefit from the vaccine without any adjuvant.

Interestingly, when fish were dip-immunized with FKC vaccine at dose  $1 \times 10^6$  4cells/ml for 1 minute, a considerable RPS (40.43-45.89%) were obtained during month post-vaccination. Our further works using higher concentration of FKC (i.e.  $1.4 \times 10^9$  cells/ml) vaccine and longer time of administration (short bath for 5 minutes) resulted in a quite higher protection up to 70% (data not shown). Therefore, in aquaculture practice it is recommended to vaccinate trout with FKC vaccine at concentration  $>1 \times 10^9$  cells/ml as a short bath for a period  $>5$  minutes. Oral vaccination of trout with FKC vaccine with or without ECP was insufficiently efficacious to *S. iniae*. However, this route of vaccination could be used as a booster particularly in case of streptococcosis in which may require a booster immunization. This is particularly feasible when fish are vaccinated by immersion route.

## Acknowledgement

This work was partly financially supported by the Research Council of University of Tehran. We thank the staff of Aquatic Animal Health Department for their assistance.

## References

- Akhlaghi, M.; Munday, B.L. and Whittington, R.J. , 1996. Comparison of passive and active immunization of fish against streptococcosis (enterococcosis). *Journal of Fish Disease*. 19:251-258.
- Chang, P.H.; Lin, C.W. and Lee Y.C. , 2002. *Lactococcus garvieae* infection of cultured rainbow trout, *Oncorhynchus mykiss*, in Taiwan and associated biophysical characteristics and histopathology. *Bulletin of the European Association of Fish Pathologists*. 22:319-327.
- Chen, S.C.; Liaw, L.L.; Su H.Y.; Ko, S.C.; Wu, C.Y. and Channg. H.C. , 2002. *Lactococcus garvieae*, a cause of disease in grey mullet, *Mugil cephalus*, L. in Taiwan. *Journal of Fish Disease*. 25(12):727-733.
- Eldar, A.; Horovitz, A. and Bercovier, H. , 1997. Development and efficacy of a vaccine against *Streptococcus iniae* infection in farmed rainbow trout. *Veterinary Immunology and Immunopathology*. 56:175-183.
- Ellis, A.E. , 1988. *Fish Vaccination*. Academic Press, London, UK. pp.2-20.
- Evans, J.J.; Klesius, P.H. and Shoemaker, C.A. , 2004. Efficacy of *Streptococcus agalactiae* (group B) vaccine in tilapia (*O. niloticus*) by intraperitoneal and bath immersion administration. *Vaccine*. 22:3769-3773.
- Evans, J.J.; Klesius, P.H.; Gilbert, P.M.; Shoemaker, C.A.; Al-Sarawi, M.A. and Landsberg, J.L. , 2002. Characterization of B-haemolytic group B *Streptococcus agalactiae* in cultured seabream, *Sparus auratus*, L. and wild mullet, *Liza kulnzingeri*, (Day) in Kuwait. *Journal of Fish Diseases*. 25(9):505-515.
- Iida, T. ; Wakabayashi, H. and Egusa, S. , 1981. Vaccination for control of streptococcal disease in cultured yellowtail. *Fish Pathology*. 16:201-206.
- Kelsius, P.H.; Shoemaker, C.A. and Evans, J.J. , 1999. Efficacy of a killed *Streptococcus iniae* vaccine in tilapia. *Bulletin of the European Association of Fish Pathologists*. 19:39-41.
- Kelsius, P.H.; Shoemaker, C.A. and Evans, J.J. , 2000. Efficacy of single and combined *Streptococcus iniae* isolate vaccine administered intraperitoneal and intramuscular routes in tilapia (*O. niloticus*). *Aquaculture*. 188:237-246.

- Kusuda, R. and Salati, F. , 1999.** *Enterococcus seriolicida* and *Streptococcus iniae*. In: Woo PTK, Bruno DW, eds. Fish Diseases and Disorders: Viral, Bacterial and Fungal Infections, CAB International. 3:303-317.
- Roberson, B.S. , 1990.** Bacterial agglutination. In: J.S. Stolen; T.C. Fletcher; D.P. Anderson; B.S. Roberson and W.B. Van Muiswinkel eds. Techniques in Fish Immunology. SOS Publications. pp.81-87.
- Romalde, J.L. and Toranzo, A.E. , 2002.** Molecular approaches for the study and diagnosis of salmonid streptococcosis. In: C.O. Cunningham ed. Molecular Diagnosis of Salmonid Diseases. Kluwer Academic Publishers, London, UK. 3:211-233.
- Romalde, J.L.; Magarinos, B. and Toranzo, A.E. , 1999.** Prevention of streptococcosis in turbot by intraperitoneal vaccination: a review, Applied Ichthyology. 15:153-158.
- Schmidtke, L.M. and Carson, J. , 1999.** Induction, characterization and pathogenicity in rainbow trout (*Oncorhynchus mykiss*) of *Lactococcus garvieae* L-forms. Veterinary Microbiology. 69:287-300.
- Sakai, M.; Austin, S. and Kobayashi, M. , 1989.** Protective immune response in rainbow trout (*O. mykiss*) vaccinated with B-haemolytic streptococcal bacterin. Fish Pathology. 24:169-173.
- Shelby, R.B.; Klesius, P.H.; Shoemaker, C.A. and Evans, J.J. , 2002.** Passive immunization of tilapia, *Oreochromis niloticus*, with anti-*Streptococcus iniae* whole sera. Journal of Fish Disease. 25:1-6.
- Soltani, M.; Jamshidi, Sh. and Sharifpour, I. , 2005.** Streptococcosis caused by *Streptococcus iniae* in farmed rainbow trout (*O. mykiss*) in Iran: Biophysical characteristics and pathogenesis. Bulletin of the European Association of Fish Pathologists. 25:95-106.
- Toranzo, A.E.; Devesa, S.; Romalde, J.L.; Lamas, J.; Riaza, A. and Lero, J. , 1995.** Efficacy of intraperitoneal and immersion vaccination against *Enterococcus* sp. Infection in turbot. Aquaculture. 134:17-27.