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Variations in *Streptococcus iniae* Vaccine Efficacy among Ornamental Cyprinids

Riccardo Russo* • **Roy P. E. Yanong**

Tropical Aquaculture Laboratory, Department of Fisheries and Aquatic Sciences, Institute of Food and Agricultural Sciences, University of Florida, 1408 24th St. SE, Ruskin, Florida 33570, USA

Corresponding author: * **ric.russo@ars.usda.gov**, **ricardorusso@hotmail.com**

ABSTRACT

Two formulations, with oil or aluminum salt as adjuvant, of an autogenous vaccine against *Streptococcus iniae* were tested in juvenile and adult rainbow (*Epalzeorhynchos erythrurus*) and red tail black sharks (*E. bicolor*, RTB shark); in juvenile blue shark (a phenotypic variant of rainbow shark created by artificial selection); and in adult rosy barbs (*Barbus conchonius*) to determine if the same vaccine could be used in closely related fish. Juvenile and adult fish were acclimated in the experimental systems for one week, and then vaccinated by intracoelomic injection. After 3 weeks, fish were challenged by intracoelomic injection with 1.5×10^5 CFU *S. iniae*/fish and mortalities recorded for 12 days. Intraspecific and interspecific differences in vaccine efficacy were observed. Both vaccine formulations were more efficacious ($p < 0.05$) in adult RTB (the Relative Percent Survival (RPS) was 82 with the oil and aluminum formulation) and rainbow sharks (the RPS was 23 and 64 with the oil and aluminum formulation) than in juvenile RTB (the RPS was 35 with the aluminum formulation) and rainbow sharks (the RPS was 11 and 8 with the oil and aluminum formulation). Both vaccine formulations were also more efficacious in juvenile and adult RTB sharks than in juvenile and adult rainbow sharks. The vaccine efficacy in juvenile blue sharks (the RPS was 10) was significantly higher ($p < 0.05$) than the protection observed in juvenile rainbow shark (the RPS was 0) when fish were challenged with 3×10^5 CFU *S. iniae*/fish. The vaccine was most efficacious in rosy barb indicating the possibility to use the same vaccine formulation for additional species of ornamental fish (the RPS was 91 and 79 with the oil and aluminum formulation).

Keywords: cyprinid, *Epalzeorhynchos* sp., ornamental fish, red-tail black shark, vaccine

INTRODUCTION

Throughout the world streptococcal infections cause significant losses in fish production, both in closed systems and in ponds (Perera *et al.* 1994; Bercovier *et al.* 1997; Shoemaker and Klesius 1997; Zlotkin *et al.* 1998; Bromage *et al.* 1999; Eldar *et al.* 1999; Colorni *et al.* 2002; Klesius *et al.* 2006; Agens and Barnes 2007; Camus *et al.* 2008; Nawawi *et al.* 2008; Park *et al.* 2008; Zhou *et al.* 2008). Streptococcosis in fish is a systemic disease associated with a strong inflammatory response by the host. External hemorrhagic areas, mainly around the base of and dorso-laterally adjacent to the pectoral fins, exophthalmia, corneal opacity, and intraocular and periorbital hemorrhages are classical signs of streptococci infections (Ferguson *et al.* 1994; Perera *et al.* 1994, 1998; Soltani *et al.* 2005; Russo *et al.* 2006; Agens and Barnes 2007; Camus *et al.* 2008; Zhou *et al.* 2008). It is a common feature in *Streptococcus iniae* infections that fish swim in a characteristic circular spinning pattern that might be correlated with the observed massive infiltration of bacteria in the brain and subsequent inflammation and damage of the brain tissue (Ferguson *et al.* 1994; Neely *et al.* 2002).

The most commonly antibiotic used for treating streptococcosis is erythromycin (Noga 1996; Stoffregen *et al.* 1996; Treves-Brown 2000; Agens and Barnes 2007). Amoxicillin (Darwish and Ismaiel 2003; Darwish and Hobbs 2005) and florfenicol (Yanong *et al.* 2005; Darwish 2007) have also showed promising results in treating *Streptococcus* spp. infections. However, environmental and regulatory concerns may decrease the possibility of antibiotic applications due to the accumulation of these compounds in the environment and to the risk of selecting for drug-resistant strains of pathogens. Vaccination of adult and juvenile fish, if effective, would help to decrease labor and drug expenses. For these reasons, several studies have tried to develop specific streptococcal vaccines for the most common farmed fish (Bercovier *et al.* 1997; Eldar *et al.* 1997; Klesius *et al.* 1999, 2000; Bachrach *et al.* 2001; Shelby *et al.* 2002; Evans *et al.* 2004; Buchanan *et al.* 2005; Pasnik *et al.* 2005a, 2005b; Delamare-Debouteville *et al.* 2006; Evans *et al.* 2006; Klesius *et al.* 2006; Locke *et al.* 2008).

Sharks and barbs are species economically important for the Florida ornamental fish industry (pers. comm., the Florida Tropical Fish Farm Association). Sharks are particularly susceptible to streptococcosis and juveniles appear most vulnerable, especially after harvest from ponds (mortality rates >80% have been commonly observed on farms) (pers. comm., local fish farmers). In a previous study, rainbow sharks (*Epalzeorhynchos erythrurus*) were observed to be more susceptible to *S. iniae* than red-tail black shark (*E. bicolor*, RTB shark) (Russo *et al.* 2006). In addition, aquaculture farmers have reported that blue shark, artificially selected from repetitive inbreeding of rainbow shark, is much more resistant to *S. iniae* than rainbow shark. The goals of this research were to test (1) the efficacy of an autogenous vaccine against *S. iniae* in juvenile RTB and rainbow sharks (RS), (2) whether or not a difference existed in the vaccine efficacy among juvenile and adult RTB and RS, (3) if there was a difference in susceptibility to *S. iniae* between rainbow, RTB and blue sharks (BS), and (4) if the vaccine could be used in other ornamental Cyprinids, such as rosy barb (RB) (*Barbus conchonius*). Data on the protection conferred by the vaccine to adult RTB sharks 8 months after vaccination were also collected.

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MATERIALS AND METHODS

Experimental animals and systems

The research was conducted at the Tropical Aquaculture Laboratory, University of Florida (Ruskin, FL). Experiments were conducted using clinically healthy juvenile RTB sharks (1.1 ± 0.4 g, 5.1 ± 0.4 cm total length (TL)), rainbow sharks (RS) (1.1 ± 0.1 g, 5.0 ± 0.2 cm TL), blue sharks (BS) (1.4 ± 0.2 g, 5.2 ± 0.6 cm TL), and adult rosy barbs (RB) (1.0 ± 0.2 g, 4.3 ± 0.4 cm TL). Adult RTB sharks (21.2 ± 5.7 g, 12 ± 0.8 cm TL) and RS (20.4 ± 4.2 g, 10.2 ± 0.5 cm TL) were also used in experiments. Fish were obtained from a local ornamental fish farm in Hillsborough County, FL. At the farm, fish were kept in a flow through system in 3,000 L vats or in ponds; in the laboratory fish were kept in two recirculating systems with 25 tanks of 38 L volume. Tanks were randomly assigned to treatments, 3 or 4 replicates for treatments were used, and each tank held 20 or 25 juvenile fish or 12 adult fish (**Tables 1-3**). Water temperature, ammonia, and nitrite in the systems were measured daily; alkalinity, hardness, and pH, once per week. The water in the system was maintained at 25.5 ± 0.6°C with a 12: 12 h light: dark photoperiod. Ammonia and nitrite concentrations were at or near 0 mg/L. Alkalinity and hardness were 110 ± 10 and 115 ± 15 mg/L, respectively; pH was 7.5. To verify the *S. iniae*-free status of the group, brain and kidney tissues were sampled from 10 representative fish before each experiment. The samples were spread on tryptic soy agar (TSA) with 5% sheep's blood (Physician's Laboratory Supply, Troy, Michigan) and incubated at 30°C for 48 h. No bacterial growth was observed in any cultured fish.

Bacterial strain and vaccine production

The strain of *S. iniae* used in these experiments was isolated from clinically diseased and moribund rainbow sharks submitted to our laboratory from the same local ornamental fish farm in Hillsborough County (FL). After necropsy and bacterial culture, *S. iniae* infection was determined to be the cause of the mortalities. Bacterial identification was obtained with the BIOLOG MicroLog3 version 4.00 System (Biolog, Inc., Hayward, California) and with standard microbiological tests as described previously (Russo *et al.* 2006). Single representative colonies from the original plates were utilized to prepare a stock broth culture. The bacteria were first purified by subculture in BBL TSA II (Physician's Laboratory Supply, Troy, Michigan) at 30°C for 24 h. The resultant purified colonies were grown for 18-24 h at 30°C in two 250 mL flasks of brain heart infusion (BHI) broth (Physician's Laboratory Supply, Troy, Michigan) enriched with 1% (v/v) sterile bovine serum (Fisher Scientific, Pittsburg, Pennsylvania). For calculating the bacterial concentration, 1 mL BHI broth culture was used for preparing serial dilutions in 9 mL saline solution; subsequently 1 mL of each dilution solution was spread in a BBL TSA II agar plate and cultured at 30°C for 24 h. After incubation, the average culture count of the BHI broth was 3×10^7 colony forming units (CFU)/mL. The broth cultures were then mixed 1:1 with sterile evaporated skimmed milk and transferred to 250 cryovials of 2.0 mL volume (VWR International, West Chester, Pennsylvania). All the aliquots were immediately frozen and stored at –70°C. For each experiment, one frozen cryovial of 2.0 mL volume was used to start the *S. iniae* culture used for challenging the fish. The cryovial was first defrosted at room temperature, and then used to prepare a 250 mL BHI broth. The broth was grown for 18-24 h at 30°C. The bacterial concentration was calculated using the protocol described above. Sterile BHI broth was used to prepare dilutions of the bacterial culture.

The vaccine used in this study was a formalin-killed bacterin produced by Novartis-Aqua Health, Ltd. (Charlottetown, Prince Edward Island, Canada) with our strain of *S. iniae*. The vaccine was produced by proprietary fermentation and downstream-processing methods. Formalin was removed by distillation. Aluminum salt or oil was used as adjuvant.

Determination of vaccine volume to be used in injection and bath immersion trials

Juvenile red-tail black sharks (n=700) were vaccinated by intracoelomic injection with different volumes of the aluminum or oil vaccine formulation to determine which dose of vaccine should be used for vaccination of juvenile fish. Twenty-five fish per four replicate tanks were vaccinated by intracoelomic injection with either 0.01, 0.03, 0.04, or 0.06 mL of the aluminum formulation or with 0.01, or 0.03 mL of the oil vaccine formulation. The control group (n=100) was injected with 0.05 mL of sterile BHI (same volume used for the bacterial challenge) (**Table 1**). Bath immersion of 100 RTB sharks and RS using the aluminum vaccine formulation was also used to determine the efficacy of this route of vaccine administration. Four groups of 25 fish each were vaccinated with the aluminum formulation by bath: fish were immersed for 1 min in a vaccine dilution of 1:4 for a total volume of 1 L (**Tables 1, 3**).

Protocols for vaccination and bacterial challenge of juvenile fishes

Additional vaccine trials were conducted in juvenile RB and in RTB, RS and BS as described below (**Tables 2, 3**). The same vaccination and challenge protocols were used in all experiments with juvenile sharks or barbs. Fish were challenged with a high dose of bacteria give dose to mimic the high mortalities (80-100%) observed in the farms during the most severe bacterial outbreaks and, in this way, we were able to observe the efficacy of the vaccine in these conditions. Fish were vaccinated seven days after introduction into tanks. Fish were anesthetized with 100 mg/L of buffered MS-222 (Tricaine-S, Western Chemical, Ferndale, WA) and then vaccinated by intracoelomic injection. In all of the following experiments, fish were injected with 0.04 mL of vaccine; the control groups were injected with 0.04 mL of sterile BHI. After 21 days, fish were challenged with 1.5×10^5 *S. iniae* CFU/fish by an intracoelomic injection of 0.05 mL of bacterial culture. Mortality was recorded for 12 days after challenge. During this period, dead fish were removed 3 times daily. Brain and kidney tissue of 70–80% of the moribund or dead fish were cultured to attempt to verify the cause of mortality. *S. iniae* was identified from the bacterial cultures using the methods previously described (Russo *et al*. 2006).

Protocols for vaccination and bacterial challenge of adult RTB sharks and rainbow sharks

Adult RTB and RS were harvested from ponds and stocked in 700 L flow through vats. Three vats for each vaccine treatment per fish species were stocked with 60 fish/vat. Fish were acclimatized for a week and then injected with 0.1 mL of vaccine. The control group was injected with 0.1 mL of sterile BHI. One day before the challenge, corresponding to 21 days post-vaccination, fish were moved to the systems in our laboratory which had similar water quality to that of the farm. Fish could not be challenged at the farm for biosecurity reasons. Four tanks were randomly assigned for controls and each vaccine treatment. Each tank was stocked with 12 fish each of each fish species. After 1 day of acclimation, adult RTB and RS sharks were challenged with 1.5×10^5 *S. iniae* CFU/fish by an intracoelomic injection of 0.05 mL bacterial culture on day 21 post-vaccination. Adult RTB sharks were also challenged as above eight months post vaccination. Mortality was recorded for 12 days after challenge (**Table 3**). During this period, dead fish were removed 3 times at day and the brain and kidney tissue of 70–80% moribund or dead fish were cultured to verify the presence of *S. iniae.*

An additional 36 adult RTB shark were injected at the farm with either the aluminum or oil formulation or BHI to serve as controls and then moved into two outdoor ponds. The fish were harvested from the ponds after 8 months and 12 fish each placed into four replicated tanks in the laboratory where they were challenged together with other adult RTB shark vaccinated a month earlier. Four tanks were randomly assigned for each treatment.

Statistical analysis

ANOVA and Tukey's *post hoc* test were run for each experiment

using the statistical program SPSS 12.0 (SPSS Inc., Chicago, IL). An arcsine (square root) transformation was performed on the mortality data expressed as percentage. Probabilities lower than 0.05 ($p < 0.05$) were considered to be significant. Relative percent survival (RPS) was calculated with the formula:

$$\text{RPS} = 1 - \frac{[(\text{treatment mortality}\,(\%))]}{\text{control mortality}\,(\%)} \times 100$$

RESULTS AND DISCUSSION

Clinical signs in infected fish

In all experiments the fish showed similar clinical signs. Moribund sharks or barbs were darker, lethargic, and showed the characteristic circular swimming pattern. Hemorrhages were observed on the ventral side of the body, on the head, and at the base of the pelvic and pectoral fins. Exophthalmia was observed in a low percentage of fish.

Determination of optimal vaccine volume in juvenile RTB shark

In the period post vaccination and pre-challenge a significant higher percent mortality (19 ± 4) than the control group (0 ± 0) was observed only in fish injected with 0.6 mL of the aluminum formulation (**Table 1**). However, the percent mortality (30 ± 7) and relative percent survival (RPS) (69) in this treatment was significantly lower than mortality in controls group (98 ± 3) and in other treatment fish injected with lower volumes of vaccine following bacterial challenge. Injected fish, regardless of the vaccine formulation used or the volume injected, were significantly lower than control fish mortality after challenge. However, for the same volume of injected vaccine, the mortality observed in fish administered the aluminum formulation (54-60%) was significantly lower than that observed in fish injected with the oil formulation (75-84%). The percent mortality of fish vaccinated by bath administration of the aluminum formulation (96 ± 5) was not significantly different from control mortality following bacterial challenge.

Vaccine efficacy in juvenile RTB sharks, RS, BS and RB

The percent mortality in RS and BS administered 0.04 mL aluminum vaccine formulation and challenged with 1.5×10^5 *S. iniae* CFU/fish by an intracoelomic injection of 0.05 mL bacterial culture on day 21 post-vaccination (Trial 1) was not significantly different than mortality in controls (**Table 2**). The percent mortality of vaccinated RTB sharks (69 ± 7) was significantly lower than the mortality of the control group (87 ± 2). Likewise, significantly lower percent mortality was noted between controls (100 ± 0) and RTB sharks (86 ± 6) and BS (90 ± 8) administered 0.04 mL aluminum vaccine formulation and challenged with 3.0×10^5 *S. iniae* CFU/fish by an intracoelomic injection of 0.05 mL bacterial culture on day 21 post-vaccination (Trial 2; **Table 2**). No significant differences in mortality were observed between controls and RS administered the higher *S. iniae* challenge dose. The percent mortality of the RB vaccinated with the vaccine with aluminum (21 ± 1) or oil (9 ± 8) as adjuvant and challenged with 1.5×10^5 *S. iniae* CFU/fish by an intracoelomic injection of 0.05 mL bacterial culture on day 21 post-vaccination (Trial 3; **Table 2**) was significant lower than the controls (100 ± 0). The greatest RPS values among all treatments and fish species were noted in RB vaccinated with either oil (91) or aluminum formulations (79).

Vaccine efficacy in juvenile and adult RS and RTB sharks

No mortalities were recorded in either RS or RTB sharks after vaccination, but 10% of the RS injected with the aluminum formulation displayed hemorrhagic areas in the ventral part of the body, and around the base of the pectoral and pelvic fins. The necropsy and bacterial cultures performed on four of these fish gave negative results for any bacterial or parasitic infections, and none of the remaining fish died before challenge with *S. iniae*. None of the RTB sharks presented similar clinical signs.

The percent mortality of juvenile RS vaccinated by injection with the aluminum or oil formulations (92 ± 7 and 89 ± 9 mortality respectively) was significantly lower than the controls (**Table 3**). The percent mortality of juvenile

Table 1 Average percent mortality ± standard deviation (SD) and relative percent survival (RPS) recorded in juvenile red tail black (RTB) shark injected with different volumes (mL) of *S. iniae* vaccine with aluminum or oil adjuvant or vaccinated by bath with the aluminum formulation 21 days after vaccination and for 12 days after the bacterial challenge with 1.5×10^5 bacteria/fish. *

Treatment	Vaccine route	Vaccine volume	Average mortality (% ± SD)		RPS
			21 d after vaccination and before bacterial challenge	12 d after bacterial challenge	
Control	Injection	0.04	0 ± 0 a	98 ± 3 c	
Oil	Injection	0.01	0 ± 0 a	84 ± 7 d	14
Oil	Injection	0.03	5 ± 5 a	75 ± 7 d	23
Aluminum	Injection	0.01	0 ± 0 a	60 ± 11 e	39
Aluminum	Injection	0.03	1 ± 2 a	54 ± 3 e	45
Aluminum	Injection	0.04	1 ± 2 a	52 ± 5 e	47
Aluminum	Injection	0.06	19 ± 4 b	30 ± 7 f	69
Aluminum	Bath	1:4 vaccine dilution	0 ± 0 a	96 ± 5 c	2

*The letters in the columns represent difference in significance level ($p < 0.05$) among treatments based on the results of the ANOVA and Tukey's test. Four replicates were used per treatment, and each tank was stocked with 25 fish for a total number of 100 fish for treatment. Control fish administered 0.4 mL BHI. RPS = Relative Percent Survival

Table 2 Average percent mortality ± standard deviation (SD) and relative percent survival (RPS) recorded in juvenile red tail black (RTB) sharks, rainbow sharks (RS), blue sharks (BS), and rosy barbs (RB) injected with vaccine with aluminum or oil adjuvant 21 days after vaccination and for 12 days after the *S. iniae* bacterial challenge with 1.5×10^5 bacteria/fish (Trials 1 and 3) or 3.0 x 10^5 bacteria/fish (Trial 2). *

Treatment	Vaccine Route	Average percent mortality ± SD and (RPS) 12 d after bacterial challenge						
		RTB		RS		BS		RB
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 3
Control	Injection	87 ± 2 a	100 ± 0 a	100 ± 0 c	100 ± 0 d	77 ± 8 ab	100 ± 0 d	100 ± 0 f
Oil	Injection	Not done	Not done	Not done	Not done	Not done	Not done	9 ± 8 g (91)
Aluminum	Injection	69 ± 7 b (21)	86 ± 6 e (14)	100 ± 0 c (0)	100 ± 0 d (0)	78 ± 8 ab (0)	90 ± 8 e (10)	21 ± 1 g (79)

*The letters in the columns represent difference in significance level ($p < 0.05$) among treatments based on the results of the ANOVA and Tukey's test. Four replicates of 20 fish each were used per treatment in trial 1 and 3 (n = 80), and three replicates of 20 fish each (n = 60) were used per treatment in trial 2. Treatment fish were administered 0.04 mL vaccine and controls were administered 0.04 mL BHI. RPS = Relative Percent Survival

Table 3 Average percent mortality ± standard deviation (SD) and relative percent survival (RPS) recorded in juvenile red tail black (RTB) and rainbow shark (RS) injected with vaccine with aluminum or oil adjuvant or vaccinated by bath with the aluminum formulation 21 days after vaccination and for 12 days after the bacterial challenge with 1.5×10^5 bacteria/fish. *

Treatment	Vaccine Route	Average percent mortality ± SD and (RPS) 12 d after bacterial challenge			
		RTB		RS	
		Juvenile	Adult	Juvenile	Adult
Control	Injection	100 ± 0 a	100 ± 0 a	100 ± 0 a	98 ± 2 a
Oil	Injection	Not done	18 ± 23 d (82)	89 ± 9 b (11)	75 ± 6 c (23)
Aluminum	Injection	65 ± 2 c (35)	18 ± 18 d (82)	92 ± 7 b (8)	35 ± 5 b (64)
Aluminum	Bath	Not done	Not done	100 ± 0 a (0)	Not done
		Fish challenged 8 months after vaccination			
Oil	Injection	Not done	71 ± 9 e (29)	Not done	Not done
Aluminum	Injection	Not done	72 ± 17 e (28)	Not done	Not done

*The letters in the columns represent difference in significance level ($p < 0.05$) among treatments based on the results of the ANOVA and Tukey's test. For juvenile RTB and RS shark four replicates were used per treatment, and each tank was stocked with 25 fish (n = 100); for adult RTB and RS shark 4 replicates were used and each tank was stocked with 12 fish (n = 48). Juvenile fish were administered 0.04 mL vaccine and control fish were administered 0.04 mL BHI; adult fish were administered 0.1 mL vaccine and control fish were administered 0.1 mL BHI. RPS= Relative Percent Survival

RTB shark vaccinated by injection with the aluminum formulation (65 ± 2) was significantly lower than the control RTB (100 ± 0) or RS vaccinated with the aluminum formulation by injection. Bacterial challenge of RS showed no significant differences in percent mortality among fish vaccinated by bath and the control group.

The percent mortalities of adult RTB sharks (**Table 3**) vaccinated for one month with the aluminum formulations (18 ± 18) and oil formulation (18 ± 23) were similar and significantly lower than the controls (100 ± 0). The RPS values (82) did not differ between the two vaccine preparations. Likewise, the percent mortalities of adult RS (**Table 3**) vaccinated with the aluminum formulation (35 ± 5) and oil formulation (75 ± 6) were significantly lower than the control RS (98 ± 2), although the aluminum formulation gave the greatest RPS value (64).

The percent mortality of RTB sharks vaccinated with the aluminum formulation (72 ± 17) or oil formulation (71 ± 9) for 8 months before the bacterial challenge (**Table 3**) were significantly higher than the mortalities of RTB vaccinated only one month before bacterial challenge (18%) although significantly lower than control RTB sharks.

DISCUSSION

These studies provide a foundation for the development of a more efficacious vaccine against *S. iniae* for ornamental sharks. The observed results are useful for setting a baseline of the level of protection given by this type of vaccine, and as a starting point for improvement and development of future vaccines. In all experiments, moribund sharks showed classical signs of streptococcosis, such as darkening of the skin, lethargy, presence of hemorrhagic areas on the ventral side of the body, on the head, and at the base of the pelvic and pectoral fins, and exophthalmia (Ferguson *et al.* 1994; Eldar *et al.* 1995; Perera *et al*. 1994, 1998; Neely *et al.* 2002; Soltani *et al*. 2005; Russo *et al.* 2006; Agens and Barnes 2007; Camus *et al.* 2008; Zhou *et al.* 2008). Fish also showed the characteristic circular swimming pattern. In this study, only intracoelomic injection of the vaccines provided protection (**Table 1**), while the bath route of vaccine administration was not efficacious, as observed in other studies (Midtlyng *et al.* 1996a, 1996b). The aluminum vaccine formulation seemed to be more efficacious than the oil formulation when administered to adult RS but no differences were observed between these treatment formulations in adult RTB sharks. In rosy barb (RB), both the aluminum and oil formulations provided superior protection, (RPS were 79 and 89) than the aluminum formulation in RTB sharks, RS and BS (the RPS were lower of 21, and for RS it was 0 in both trials; **Table 2**). Interestingly, in salmonids, oil adjuvant vaccines are more effective than aluminum formulations (Midtlyng *et al*. 1996a, 1996b; Nordmo and Ramstad 1997). It is important to consider this difference in adjuvants efficacy among species for the development of new vaccines.

As seen from the results reported in **Tables 2** and **3**, both aluminum and oil vaccine formulations were more efficacious in adult RTB and RS than in juveniles, but it is difficult to make a true comparison due to the different doses of vaccine injected in the juvenile and adult fishes. These results suggest that in the future a better vaccine formulation, possibly with a higher concentration of antigens or upregulation of select antigens should be developed at least for the juvenile fish. The use of vaccines that confer low or moderate protection may still be economically beneficial because these vaccines may slow down or stop the rate of disease transmission between infected and non-infected individuals (Anderson and May 1985; Waltner-Toews 1989). A reduction in the rate of transmission may be helpful especially when the production periods are not too long, as in aquaculture where the production period is generally of one or two years. Although not determined here, vaccines can also increase resistance to other disease or stressful situations, improved growth rate and feed efficiency so as to make their use economically effective even if there is no decrease in mortality (Mitchell 1997, 1999). A certain level of protection, even if low, was still present 8 months after vaccination in RTB sharks (**Table 4**). Due to pond availability at the farm and to the high economic value of broodstock fish, we were not able to obtain more broodstock shark to determine a protection/time relation. However, these results are important because they represent a step forward in developing a vaccine schedule for broodstock sharks. A booster injection of vaccine a few weeks after the first vaccination might help to increase the efficacy of the vaccine, and will be considered in future experiments.

We observed a difference in resistance to *S. iniae* infections and vaccine efficacy among RTB, rainbow, blue sharks, and rosy barb. In all experiments, the efficacy of the aluminum and oil vaccine formulations was higher in RTB shark than in rainbow shark. Similarly, in a previous study aimed to develop a challenge model against *S. iniae* in these two shark species (Russo *et al*. 2006), we observed that rainbow shark challenged with different doses of bacteria were more susceptible to *S. iniae* infection than RTB shark injected with the same bacterial concentration. Mortalities occurred faster in the rainbow shark population, and deaths were no longer observed 4 days after the bacterial challenge. In contrast, deaths were still observed 11 days post-challenge in the RTB shark. The protection level conferred to rosy barb was closer to the protection observed in RTB adult sharks and exceeded the protection observed in all species of juvenile sharks tested.

This research demonstrated that differences in resistance to disease and differences in vaccine efficacy might be present in the same species of fish among different phenotypes from the same population, and among closely related species. Disease resistance due to genetic variation, as presumptively observed in this research, is an important factor that should be considered by every aquaculture facility to increase productivity and decrease production expen-

ses. Several other studies with fish have observed differences in disease resistance among different populations of the same species, or among closely related species. For example, genetic variations in resistance to the viral haemorrhagic septicemia virus (VHSV) were observed in rainbow trout (*Oncorhynchus mykiss* Walbaum) (Dorson *et al*. 1995; Quillet *et al.* 2001), and virus-resistant strains of fish were selected for breeding. Similar research was conducted using lines and their hybrids of common carp (*Cyprinus carpio* Linnaeus) to examine genetic differences in resistance against *Aeromonas salmonicida* (Wiegertjes *et al*. 1995a) and *Trypanoplasma borreli* (Wiegertjes *et al.* 1995b). Genetic variations in resistance to *Cryptobia salmositica* (Chin *et al*. 2004) and to *Gyrodactylus salaris* (Dalgaard *et al*. 2004) were observed among populations of Atlantic salmon. Interspecific genetic variations in resistance to VHSV, infectious haematopoietic necrosis virus (IHNV), and infectious pancreatic necrosis virus (IPNV) were also observed among rainbow trout, arctic char (*Salvelinus alpinus* Linnaeus), and lake trout (*S. namaycush* Walbaum) and their hybrids (Dorson *et al.* 1991). Genetic variation in resistance to the Gram negative bacteria *Edwardsiella ictaluri* were also reported for channel catfish, *Ictalurus punctatus* (Booth and Bilodeau-Bourgeois 2008).

CONCLUSIONS AND FUTURE PERSPECTIVES

The results of this research have achieved the development of challenge models and vaccine efficacy tests for two ornamental fish, which may also be applied for future studies on new vaccines for other species of ornamental fish. As has been described previously, low efficacy may be observed in the first attempts to develop new vaccines, but the results of the first attempts are important in establishing a baseline level of protection and for allowing improvement and development of future vaccines. This research provides a good example of how the protection level conferred by vaccines can be variable. Especially in the field, there is often a misunderstanding of what is meant by "vaccine efficacy" and the fact that the results of vaccination procedures can be uncertain. It is not uncommon that a group of vaccinated fish has the same mortality rate as control fish. Vaccines help to prevent diseases and reduce outbreak severity, but do not prevent infections. The effect of vaccination is usually observed over a long period of time and consists of decreasing or eliminating disease incidence. For this reason vaccine efficacy is more complicated to determine than disease prevention. Vaccines can also increase resistance to other disease or stress situations, and indirectly improve growth rate and feed efficiency, so as to make their use economically effective even if there is no decrease in mortality. The efficacy of vaccines is also influenced by many factors (genetic, nutrition quality, stress, water temperature, handling, etc.), and most of them are either uncontrollable or underestimated. For this reason, although great differences in results might be seen among facilities, this does not necessarily imply that the vaccination program is not efficient.

In this research we demonstrated the efficacy of a vaccine against *S. iniae* when adjuvants (aluminum salt or oil) were added to the basic formulation. As reported many times in the literature, adjuvants are very effective in increasing the immunostimulatory capability of vaccines due to their mechanisms of action. Unfortunately, in our studies the data indicated that only the injection route of administration was efficient in providing protection to the fish, but, for obvious reasons, this route is not very economically feasible for juvenile fish. Construction of an automated vaccine delivery system or improvement of oral delivery of vaccines using microcapsule could make vaccination by injection in juvenile fish economically feasible for the ornamental fish industry.

Under the conditions of this research, we observed a difference in vaccine efficacy among red-tail black shark, rainbow shark, and blue shark. This is one of the more interesting findings of this research. Disease resistance due to genetic variation is an important factor that should be considered for developing successful breeding programs. The selection of strains of fish less susceptible to disease results in an increase of biomass production and in a decrease of production expenses. However, because the strain of *S. iniae* used in this research was originally isolated from rainbow, this might be another possible contributing factor to the difference in disease resistance between RTB and rainbow shark.

The results of this research and of similar studies should also be used to promote the application of regular vaccination protocols in ornamental fish farms instead of relying in massive and frequent use of antibiotics. An extensive use of antibiotics may increase the risk of selecting resistant strains of bacteria in the environment. Furthermore, in addition to preventing mortalities, vaccination can be much more cost effective than antibiotics. There are several associated costs faced during and following antibiotic treatment; for example the costs associated to activities as removal of dead fish, improvement of water quality in systems where many fish died, disinfection and restocking of culture systems affected by high losses. Furthermore, one of the possible side effects of disease outbreaks is a decrease growth rate of the fish that will result in longer culture periods to reach the commercial target size of the fish.

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