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Characterization of *Streptococcus iniae* isolated from ornamental cyprinid fishes and development of challenge models

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Abstract

Streptococcus iniae was isolated from red-tail black shark(RTB shark) *Epalzeorhynchos bicolor*, and rainbow shark, *E. erythrurus* (fam: Cyprinidae), obtained from a local freshwater ornamental fish farm in Hillsborough county (FL). Darkening of the skin and lethargy were the first signs observed in infected sharks. 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 (~ 10%). Moribund fish demonstrated the characteristic spinning, swimming pattern observed during streptococcal infections. Histological analysis revealed leukocyte infiltration in the intestinal area, spleen, posterior kidney, and brain. Necrosis and tissue degeneration were observed in the same organs in addition to degeneration of the renal tubules. Gram positive cocci were isolated from the cultures of brain and kidney tissues. Bacterial identification was obtained with the BIOLOG MicroLog3 version 4.00 System (Biolog, Inc., Hayward, California) and with standard microbiological tests. *S. iniae*-free RTB and rainbow sharks were used for developing a challenge model with the isolated bacteria strain. The experiments were conducted in two separate recirculating systems each with 25 tanks, 38 L volume per tank. In all experiments, the water in the system was maintained at 25 ± 1 °C. For each treatment, there were 3 replicate tanks, each stocked with 25 fish. Tanks were randomly assigned to treatment or control groups. Sharks were challenged by intracoelomic injection of *S. iniae*, and mortality was recorded for 14 days. Moribund fish showed classical signs of streptococcosis and *S. iniae* was isolated from moribund and dead fish. Differences in susceptibility to *S. iniae* were observed between the two species. An injection of 1.5×10^4 colony forming units (CFUs)/fish caused mortality close to 70% in RTB shark, and a higher mortality rate in rainbow shark. Mortalities ceased in 11–12 days after bacterial challenge in RTB shark, and in 3–4 days in rainbow shark. In this report, the pathology of *S. iniae* infections in two ornamental freshwater sharks and the development of a challenge model are described.
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1. Introduction

Streptococcal infections in fish result in septicemic diseases that have been reported worldwide causing severe economic losses in fish production (Bercovier et al., 1997; Eldar et al., 1997; Shoemaker and Klesius,

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1997). Streptococcosis can affect wild and farmed fish in both fresh and saltwater (Perera et al., 1998; Sako, 1998; Zlotkin et al., 1998; Colorni et al., 2002). In ornamental fish, *Streptococcus* spp. have been isolated from zebra danio, *Danio rerio*, pearl danio, *D. albolineatus* (Ferguson et al., 1994), clown loaches, *Botia macracanthus*, rosy barbs, *Barbus conchonius* (Yanong and Russo, unpublished data), tetras, *Hyphessobrycon* sp., and African cichlids of the genera *Nimbochromis* and *Pelvicachromis* (Yanong and Floyd, 2002). *Streptococcus* spp. may be introduced into farms by new imported fish, especially when a real quarantine facility is missing, or by other animals, such as birds or insects, that may spread pathogens from a farm to another. Furthermore, most of the ornamental fish farms in Florida raise fish in ponds that are part of recirculating system. For this reason, once *Streptococcus* spp. is introduced into a farm, it may be very difficult to eradicate it. The possibility of streptococcal outbreaks is increased if fish are stressed, as what happens with non-optimal water temperature, low dissolved oxygen, high nitrite levels, and high culture densities (Bunch and Bejerano, 1997; Perera et al., 1997; Shoemaker et al., 2000).

Streptococcosis can be treated with antibiotics: erythromycin, the most commonly used, florfenicol and amoxicillin (Treves-Brown, 2000; Darwish and Hobbs, 2005; Yanong et al., 2005). Several vaccines have been developed against streptococci, mainly for tilapia and rainbow trout (Eldar et al., 1997; Klesius et al., 2000; Shelby et al., 2002).

Despite the fact that few cases have been reported, there is some concern about potential transmission of *S. iniae* from fish to humans. It has been suggested (Weinsten et al., 1996, 1997; Shoemaker and Klesius, 1997; Goh et al., 1998; Lau et al., 2003) that the possibility of transmission is low and mainly of concern for immunocompromised individuals. Recent reports in China suggest higher risk of *S. iniae* infections in Asian populations due to their habit of eating raw fish (Lau et al., 2003).

The purpose of this study was (1) to describe the clinical signs, pathology, and bacteriology of *Streptococcus* sp. isolated from red-tail black shark *Epalzeorhynchos bicolor* (RTB shark), and rainbow shark, *E. erythrurus* (fam: Cyprinidae), raised in local ornamental fish farm in Hillsborough County (FL); and (2) to develop a challenge model with the same bacteria for sharks. Sharks were chosen because they are economically important to Florida's freshwater ornamental fish industry, and are very susceptible to streptococcosis.

2. Materials and methods

2.1. Clinical cases

Different populations, from different facilities, of RTB and rainbow sharks were submitted to the Tropical Aquaculture Laboratory, University of Florida, Ruskin, Florida, from local ornamental fish farms in Hillsborough County (FL) for diagnostic evaluation. High mortalities (~40–60%) were recorded at a number of these farms. For each clinical cases, skin, gills, and fin biopsies and samples of internal organs were examined with light microscopy. Hematoxylin and eosin (H&E) or Gram staining of histological sections was carried out on samples of internal organs preserved in 10% neutral buffered formalin. Brain and kidney samples of moribund fish were cultured on Tryptic Soy Agar with 5% sheep's blood (Physician's Laboratory Supply, Troy, Michigan) and incubated at 30 °C for 48 h. For each clinical case, *Streptococcus iniae* was identified from bacterial cultures by (1) observation of the colony morphology in the bacterial cultures, (2) Gram stain of the bacterial culture, (3) observation under light microscopy of histological sections for Gram positive cocci in affected tissue, and (4) identification of bacterial cultures using the BIOLOG MicroLog3 version 4.00 System (Biolog, Inc., Hayward, California). Findings routinely showed presence of Gram positive cocci that were identified as *S. iniae* by the BIOLOG system.

2.2. Experimental animals and system

The development of a challenge model was conducted using two recirculating systems, each with 25 tanks, 38 L volume per tank. RTB shark (mean weight 1.3±0.4 g and TL 5.2±0.5 cm) and rainbow shark (mean weight 1.0±0.2 g and TL 4.9±0.4 cm) juveniles were obtained from a local ornamental fish farm in Hillsborough County (FL) where they were raised in ponds. Water temperature, ammonia, and nitrite in the systems were measured daily; alkalinity, hardness and pH once a week (Fish Farm Kit, Model FF-1A, Hach Co., Loveland, Colorado, USA). The water in the system was maintained at 25.5±0.6 C. Ammonia and nitrite concentrations were 0 mg/L, alkalinity 110±10 mg/L, hardness 115±15 mg/L and pH 7.5. To provide some assurance as to the *S. iniae*-free status of the fish, brain and kidney tissues were sampled from 10 representative fishes before each experiment. The samples were cultured on Tryptic Soy Agar with 5% sheep's blood (Physician's Laboratory Supply, Troy, Michigan) and incubated at 30 °C for 48 h. No bacterial growth was observed.

2.3. Bacterial strain

The strain of *S. iniae* used was an isolate from naturally infected rainbow sharks submitted to our laboratory and originating from the same ornamental fish farm in the Hillsborough County, Florida that provided the fish for these experiments. After necropsy and bacterial culture, *S. iniae* infection was determined to be the cause of the disease. Bacterial identification was obtained with the BIOLOG MicroLog3 version 4.00 System (Biolog, Inc., Hayward, California) and with standard microbiological tests (Table 1). Single representative colonies from the original plates were utilized to prepare a stock culture. The bacteria were first purified by subculture in BBL TSA II (Physician's Laboratory Supply, Troy, Michigan; Tryptic Soy Agar with 5% sheep's blood = TSA5SB) at 30 °C for 24 h. A few of the resultant purified colonies were grown for 24 h at 30 °C in two 250 mL brain heart infusion (BHI) broth (Physician's Laboratory Supply, Troy, Michigan) flasks enriched with sterile bovine serum to 1% v/v (Fisher Scientific, Pittsburgh, Pennsylvania). For calculating the bacterial concentration, 1 mL BHI broth culture was used for preparing serial dilutions in 9 mL saline solution; successively 1 mL of each dilution solution was spread on a TSA5SB plate and cultured at 30 °C for 24 h. After incubation the average culture count of the BHI broth was 3×10^7 bacteria/mL. The broth cultures were then mixed 1:1 with sterile evaporated skim milk and transferred to 250 cryovials of 2.0 mL volume. All the aliquots were immediately frozen and stored at −70 °C. For each experiment, a *S. iniae* culture was prepared using one frozen cryovial. The cryovial was first defrosted at room temperature, and then used to prepare a 250 mL BHI broth (Physician's Laboratory Supply, Troy, Michigan) incubated for 24 h at 30 °C.

Table 1
Comparison of characteristics of *S. iniae* isolated from diseased rainbow shark with strains isolated from other fish species

Test	Present study	Perera et al. (1994)	Sako (1998)
	Rainbow shark	Tilapia hybrid	Yellowtail, ayu, common mackerel, Japanese flounder
Cell morphology	Spherical, long chains	Spherical	Spherical
Gram stain	+	+	+
Growth at 10 °C	+	+	ND
Growth at 45 °C	−	+	ND
Growth in 6.5% NaCl	−	−	ND
Catalase	−	−	−
Hemolysis	Alpha/beta	Beta	Beta
Hydrogen sulfide production	−	ND	−
Esculin hydrolysis	−	ND	+
Hippurate hydrolysis	−	−	−
Voges–Proskauer	−	−	−
Acid from:			
Arabinose	+	−	−
Glucose	+	+	+
Lactose	−	−	−
Mannitol	−	+	+
Raffinose	−	−	−
Salacin	−	−	+
Sorbitol	−	−	−
Trehalose	+	+	+
D-xylose	−	−	−

Table 2
Average cumulative mortality (%) 14 days after bacterial challenge for the experiments with RTB shark

Specie	Trial	Treatment	Final mortality	Significance
	n	CFUs/fish	%±SD	
RTB shark (*E. bicolor*)	1	Control	0±0	a
		1.5×10^7	100±0	b
		1.5×10^8	100±0	b
		1.5×10^9	100±0	b
		1.5×10^{10}	100±0	b
	2	Control	0±0	c
		1.5×10^1	5±0	c
		1.5×10^2	13±6	c, d
		1.5×10^3	44±13	d, e
		1.5×10^4	65±18	e
	3	Control	0±0	f
		1.5×10^1	7±9	f
		1.5×10^2	3±5	f
		1.5×10^3	66±17	g
		1.5×10^4	75±12	g, h
		1.5×10^5	86±5	h, i

Three replicates were used per treatment and each tank was stocked with 25 fish. In the treatment column the concentration of injected *S. iniae* (bacteria/mL) used for each trial is reported, and different letters in the last column notes significant difference among groups based on the Tukey's test ($p_2=0.0017$, $p_3=3.6\times10^{-8}$, $\alpha=0.05$) are in the last column.

2.4. Experimental design

The challenge model for the RTB shark was developed based on the results from three experiments (experiments 1–3), and two other experiments were done with rainbow shark to check the validity of this

Table 3
Average cumulative mortality (%) 14 days after bacterial challenge for the two experiments with rainbow shark

Specie	Trial	Treatment	Final mortality	Significance
	n	CFUs/fish	%±SD	
Rainbow shark (*E. erythrurus*)	4	Control	0±0	a
		1.5×10^{3}	45±12	b
		1.5×10^{4}	86±5	c
		1.5×10^{5}	98±3	d
		1.5×10^{6}	100±0	d
	5	Control	2±2	e
		1.5×10^{1}	2±3	e
		1.5×10^{2}	6±2	e
		1.5×10^{3}	21±2	f
		1.5×10^{4}	79±11	g
		1.5×10^{5}	100±0	h

Three replicates were used per treatment and each tank was stocked with 25 fish. In the treatment column the concentration of injected *S. iniae* (bacteria/mL) used for each trial is reported, and different letters in the last column notes significant difference among groups based on the Tukey's test ($p_4=3.4\times10^{-12}$, $p_5=3.4\times10^{-12}$, $\alpha=0.05$) are reported in the last column.

model (experiments 4 and 5). All five experiments used the same protocol. Three tanks were randomly assigned for each treatment and for controls. Each tank was stocked with 25 fish. After a week of acclimation, fish were challenged by an intracoelomic injection of 0.05 mL of *S. iniae* culture. The control group was injected with 0.05 mL of sterile BHI. The colony forming unit (CFU) dose injected into each fish for each experiment is reported in Tables 2 and 3. For *S. iniae* doses higher than 10^7 CFU/fish, bacterial cultures were first centrifuged at 2000 IPM for 30 min and then the pellet was resuspended in sterile BHI to reach the target bacterial concentration. Fish mortality was recorded for 14 days after challenge. During this period, dead fish were removed twice daily, except in experiment 1, where dead fish were removed 3 times per day. In each experiment, representative brain and kidney cultures were taken from 70% to 80% of dead fish for each treatment to verify the cause of mortality; and 12 representative moribund fish for each treatment were selected and preserved in 10% neutral buffered formalin. Hematoxylin and eosin (H&E) or Gram staining of tissue sections was completed for a representative sample of 10 of the 12 preserved fish. *S. iniae* was identified from the bacterial cultures by (1) observation of the colony morphology in the bacterial cultures, (2) Gram stain of 50% bacterial culture for each treatment, (3) observation under light microscopy of histological sections for Gram positive cocci in affected tissue, and (4) identification of 3–4 bacterial cultures for treatment using the BIOLOG MicroLog3 version 4.00 System (Biolog, Inc., Hayward, California). Results consistently revealed the presence of Gram positive cocci that were identified as *S. iniae* by the BIOLOG system.

2.5. Statistical analysis

A one way ANOVA and Tukey's post hoc test were run for each experiment using the statistical program SPSS 12.0 (SPSS Inc., Chicago, IL). An arcsin (square root) transformation was performed on the mortality data expressed as percentage. Probabilities lower than 0.05 ($p<0.05$) were considered significant.

3. Results

Similar clinical signs were observed in naturally infected sharks submitted for diagnostic evaluation and in sharks experimentally infected with *S. iniae*. Darkening of the skin and lethargy were the first signs observed in challenged RTB and rainbow sharks. Hemorrhages were observed on the ventral side of the body and on the head, and at the base of the pelvic and pectoral fins. Exophthalmia was observed in less than 10% of fish. Moribund fish demonstrated the characteristic spinning, swimming pattern. Histological analysis revealed leukocyte infiltration in the intestinal area, spleen, posterior kidney and brain. Necrosis and tissue degeneration were observed in the same organs. No granulomas were observed in tissues. *S. iniae* was recovered from all cultured moribund or dead sharks and recognized by the white pinpoint shape of the bacterial cultures on TSA5SB by observation of gram positive cocci from these cultures, and by positive identification with the Biolog system.

In the first experiment, less than 12 h after bacterial challenge, fish began to show classical signs of streptococcosis and the first mortalities were recorded. All concentrations of *S. iniae* tested caused 100% mortality of RTB shark in the first two days after bacteria challenge (Table 2). In the second experiment (Table 2) average mortalities of 44±13% and 65±18% 14 days after bacterial challenge were observed when fish were injected with 1.5×10^3 and 1.5×10^4 CFUs, respectively. Challenge with lower doses did not cause significantly higher mortality than in the control group. In the third experiment (Table 2) average mortalities of 66 ±17%, 75±11% and 86±5%, 14 days after bacterial challenge, were observed when fish were injected with 1.5×10^3, 10^4 and 10^5 CFUs, respectively. In this same trial, challenge with lower doses did not cause significantly higher mortality than in the control group.

The results of experiments 4–5 with rainbow shark (Table 3) demonstrated that injections of 1.5×10^4 CFU/fish resulted in an average percentage mortality close to 80% in 14 days; injection of 1.5×10^3 CFU/fish caused a lower mortality and less consistently among trials; and injection of higher doses caused 100% mortality. Mortality in rainbow shark stopped after 3–4 days, except for one treatment in experiment 4.

4. Discussion

Darkening of the skin and lethargy were the first signs observed in infected RTB and rainbow sharks. Moribund and dead fish presented external hemorrhages especially around the base of the pectoral fins and over the heart, and exophthalmia. Histological examination of RTB and rainbow sharks revealed leukocyte infiltration, necrosis, and tissue degeneration of several internal organs. These observations are in accordance with what has been reported in the literature (Ferguson et al., 1994; Perera et al., 1998; Neely et al., 2002) where *S. iniae* is described to cause a systemic disease associated with a strong inflammatory response by the host. Externally, streptococcosis-induced lesions included pronounced congestion and hemorrhages, exophthalmia, corneal opacity, intra-ocular and periorbital hemorrhages. Neutrophilic and macrophage infiltration, and tissue necrosis were observed in several organs, especially in the brain, spleen, posterior kidney, liver and intestinal tract. Pathological changes associated with the brain, such as meningoencephalitis, and with the nervous system are typical of streptococcosis infections (Ferguson et al., 1994; Neely et al., 2002). Due to the high capability of spleen and posterior kidney to trap bacteria, streptococci can be commonly observed in these organs in conjunction with tissue necrosis (Ferguson et al., 1994; Perera et al., 1998).

Diseased, moribund sharks demonstrated a spinning swimming that is a common presentation of *S. iniae* infections, which might be correlated with the massive cellular infiltration observed in the brain (Ferguson et al., 1994; Perera et al., 1998). *S. iniae* has tropism for the brain, and this tropism is one of the main causes of death. The invasion rate of *S. iniae* into different organs has been carefully observed and described in yellowtail (Kusuda and Kimura, 1978; Kusuda and Kawai, 1982; Sako, 1998). In the first two days after bacterial challenge, there was an increase in bacterial count in blood, spleen, kidney and brain. Afterward, the bacterial count in the blood, spleen and kidney decreased or remained constant, while in the brain it increased until onset of mortality.

One aim of this research was to develop a challenge model against *S. iniae* in RTB and rainbow sharks; that is, to determine the lethal dose of bacteria needed to kill a predetermined percentage of fish in a fixed period of time. In most studies, a lethal dose of bacteria/CFUs that should kill 70% of the fish population in 14 days is chosen as target bacteria/CFU dose; however these numbers may vary among research groups (Kimura and Kusuda, 1979; Nordmo and Ramstad, 1997; Perera et al., 1997; Sako, 1998). Developing challenge models is one of the first steps in vaccine or pharmaceutical development for animal diseases. Fish can be infected by bath with a chosen bacteria concentration; by introduction of diseased fish or by intracoelomic or intramuscular injection. The advantage of the first two challenge techniques is that they replicate the natural route of the mucosal immunity is involved in protecting fish from the infection. The advantages of challenging fish by an intramuscular or intracoelomic injection is that this technique is more replicable and efficient than the others two (Kimura and Kusuda, 1979; Perera et al., 1997; Sako, 1998). However, in some studies no difference in mortality rate was observed at the end of the experiments in vaccinated fish between the intracoelomic and cohabitation challenge methods (Nordmo and Ramstad, 1997). In this research the intracoelomic injection was chosen as challenge route for its replicability. The results of the challenge model described in this manuscript were replicated in a subsequent investigation on the efficacy of three vaccine formulations against *S. iniae* for RTB shark and rainbow shark (Russo, 2004).

The results of these studies with RTB shark, demonstrate that a plausible challenge model against *S. iniae* by intracoelomic injection in 5 cm RTB shark kept at 25 °C is possible. In this model consistent mortality close to 70% within 14 days after bacterial challenge was seen after injecting fish with 1.5×10^4 CFUs. Fish began to show clinical signs and mortality after less than one day post-challenge. Mortality due to *S. iniae* was confirmed by identification from brain and kidney cultures of moribund and dead fish.

Experiments 2 and 3 were conducted in different seasons and using different stocks of juvenile RTB shark. Accordingly, the differences in mortality rates observed between the two experiments could be due to genetic variation or environmental factors that influenced the fish when they were raised in ponds (i.e. water temperature, stocking density, feed consumption, dissolved oxygen). Variations in mortality rates among trials are not uncommon, as observed in studies on infection of *S. iniae* in yellowtail, for example (Sako, 1998).

The results of the experiments with rainbow shark demonstrate the validity of the challenge model developed for RTB shark also for this species, even though there were some small differences. The injection of 1.5×10^4 CFU/fish caused a higher mortality rate in rainbow than in RTB shark, and in rainbow shark mortalities ended 3–4 days after challenge, and not after 11–12 days as was seen in RTB shark.

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